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## Dynamics of the host-parasite interaction: in vitro correlates of Crassostrea-induced modulation of Perkinsus marinus function

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DYNAMICS OF THE HOST-PARASITE INTERACTION: *IN VITRO* CORRELATES  
OF *CRASSOSTREA*-INDUCED MODULATION OF *PERKINSUS MARINUS*  
FUNCTION

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A Dissertation  
Presented to  
The Faculty of the School of Marine Science  
The College of William and Mary in Virginia

In Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

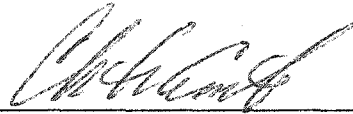
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by  
Christopher G. Earnhart

2004

## APPROVAL SHEET

This dissertation is submitted in partial fulfillment of  
the requirements for the degree of  
Doctor of Philosophy



Christopher G. Earnhart


Approved, April 16<sup>th</sup>, 2004



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## DEDICATION

To Katie for her never-ending love and encouragement, Jack for his energy and curiosity, my parents for their support through the years, and God for guiding me down this path.



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## ABSTRACT

*Perkinsus marinus* is an alveolate protozoan parasite of the eastern oyster (*Crassostrea virginica*) which is responsible for much of the decline in United States oyster populations. *Perkinsus marinus* can be cultured *in vitro*, but is rapidly attenuated in the process. Supplementation of a protein-free medium with products of *P. marinus*-susceptible (*C. virginica*) and tolerant (*C. gigas*, *C. ariakensis*) oysters altered proliferation, changed protease expression profiles in the parasite extracellular products (ECP), induced morphological forms typically seen *in vivo*, and partially reversed *in vitro* attenuation. Increased infectivity was not consistently associated with changes in cell size, cell morphology, or protease secretion, and was not related to the susceptibility of the oyster used as the supplement source. Supplements derived from dissected oyster tissues were used to determine if these changes could be differentially elicited. These supplements, with the exception of adductor muscle, reduced proliferation. Whole oyster and digestive gland/gonad supplements favored palintomic, rather than binary, fission. The total ECP protease activity was generally decreased in supplemented cultures, though gill/mantle supplemented cultures may have undergone protease induction. Zymograms indicated that the low molecular weight subset of proteases were upregulated most effectively by heart- and adductor muscle-derived supplements.

Serine proteases and other ECP proteins may be virulence factors. Attempts to create antibodies to study *P. marinus* cells and ECP have been largely unsuccessful due to poor immune responses and crossreactivity with host oyster antigens. In this study, ultrafiltration-concentrated culture supernatants containing the *P. marinus* ECP were both poorly immunogenic and toxic to experimental animals. Immunogenicity was not substantially affected by heat denaturation or proteolytic inhibition. Co-administration of ECP with oyster plasma caused a suppression in the anti-plasma antibody response with restriction of epitope recognition. Analysis of medium constituents revealed that a surfactant, Pluronic F-68 (PF68), was immunosuppressive. Although isolated protein antigens from *P. marinus* ECP remained immunosuppressive, separation of the antigens from PF68 enabled production of monoclonal antibodies. It also allowed subtractive immunization for attempted production of antibodies specific to proteins upregulated by oyster supplement exposure; however, the changes in ECP from supplemented media were either not associated with generation of novel epitopes, or those epitopes were in sub-immunogenic concentrations. Five monoclonal antibodies were created using ECP from unsupplemented medium and were used to study ECP function, regulation, and mechanism of storage and release. ECP are secreted by several pathways, including release from the cell wall and from two morphologically distinct intracellular compartments. A sandwich ELISA format allowed sensitive and precise quantification of an ECP protein with significantly reduced expression in supplemented cultures. Another antibody, which specifically bound to trophozoite and tomtom walls, was used to investigate the morphological and antigenic changes during thioglycollate-induced formation of prezoosporangia, and confirm supplement-induced formation of prezoosporangia. This antibody also labeled *P. marinus* cells in fixed oyster tissue in a species-specific manner.

DYNAMICS OF THE HOST-PARASITE INTERACTION: *IN VITRO* CORRELATES  
OF *CRASSOSTREA*-INDUCED MODULATION OF *PERKINSUS MARINUS*  
FUNCTION

## Chapter 1

### Introduction

The eastern oyster, *Crassostrea virginica*, is a bivalve mollusc of commercial, ecological, and historical importance. The species occurs in natural populations from the Canadian Maritime provinces to Texas, as well as in Panama and the Caribbean (Carlton and Mann, 1996). It is also present in introduced populations on the Pacific coast of the United States and in Hawaii (Carlton and Mann, 1996; Muñoz et al., 2003). Over the past one hundred years, the historically large populations have undergone significant decline, initially due to fishing and, later, disease pressures (Andrews, 1988; Hargis and Haven, 1988; Rothschild et al., 1994). In the 1880s, harvests of oysters in Virginia's portion of the Chesapeake Bay amounted to nearly 10 million bushels per year (Hargis and Haven, 1988; MacKenzie, 1997). Landings fell steadily over 75 years to approximately 3 million bushels per year by 1955 (Hargis and Haven, 1988; MacKenzie, 1997). This decline was likely due to depletion of existing natural oyster beds and subsequent siltation and fouling of the small amount of residual shell (Rothschild et al., 1994; MacKenzie, 1997).

During the last half of the twentieth century, oyster landings have fallen below 500,000 bushels per year due in large part to two parasitic diseases, commonly referred to as MSX and Dermo (Andrews, 1979; Andrews, 1988; Hargis and Haven, 1988; MacKenzie, 1997). MSX is caused by the haplosporidian parasite *Haplosporidium nelsoni* and has been responsible for epizootics in the mid-Atlantic states. It was initially reported as the cause of mortalities in 1957 in Delaware Bay, and two years later appeared in the Chesapeake Bay (Haskin et al., 1966; Andrews and Wood, 1967; Andrews and Castagna, 1978; Ford and Haskin, 1982). Through screening

of natural populations and selective breeding techniques, strains of oysters have since been developed with decreased susceptibility to the disease (Haskin and Ford, 1979; Ford and Haskin, 1987). Since the late 1980s, MSX has been replaced by Dermo as the most significant oyster disease (Craig et al., 1989; Burreson, 1991; Burreson and Ragone-Calvo, 1996; Chu and Volety, 1997). Dermo is caused by the protozoan parasite *Perkinsus marinus*.

*Perkinsus marinus* was first described by Mackin et al. (1950) and named *Dermocystidium marinum*, due to its perceived resemblance to fungal parasites of fish. It was later renamed *Labyrinthomyxa marina* based on observation of labyrinthulid protistan forms which were later felt to be cultural contaminants (Mackin and Ray, 1966; Perkins, 1996). In 1978, Levine proposed its placement into a new genus, *Perkinsus*, in the phylum Apicomplexa based on the observation by Perkins of a partial apical complex in the motile zoospore stage (Perkins, 1976b; Levine, 1978). Recent phylogenetic analysis using small-subunit ribosomal RNA and actin sequences has indicated that *Perkinsus* is phylogenetically basal to the apicomplexans and dinoflagellates, though it is likely more closely related to the latter (Fong et al., 1993; Goggin and Barker, 1993; Siddall et al., 1995; Reece et al., 1997; Noren et al., 1999; De La Herran et al., 2000). In 1993, it was suggested that *Perkinsus* be placed in a new subphylum, Apicomonada, under the phylum Apicomplexa (Cavalier-Smith, 1993). This was later revised, with *Perkinsus* being a member of the infrakingdom Alveolata, superphylum Miozoa, phylum Dinozoa, subphylum Protalveolata (Cavalier-Smith, 1998). Phylum Dinozoa also contains, within subphylum Dinoflagellata, the dinoflagellates (Cavalier-Smith, 1998). Noren et al. (1999) have proposed that *Perkinsus* be placed into a new phylum, Perkinsozoa.

Though *P. marinus* has likely been present in oyster populations since at least the early 1900s, in the past 15 years it has caused major oyster mortalities in Atlantic populations south of Delaware Bay (Burreson and Ragone-Calvo, 1996; Ford, 1996).

*Perkinsus marinus* occurs with highest prevalence and intensity in areas of high salinity and temperature, and the occurrence of *P. marinus* prior to the late 1980s was primarily restricted to high-salinity coastal bays south of Delaware Bay and in the Gulf of Mexico (Craig et al., 1989; Paynter and Burreson, 1991; Burreson and Ragone-Calvo, 1996). This is in agreement with laboratory findings that *P. marinus* is most infective under conditions of high salinity and temperature (Chu and Greene, 1989; Chu and LaPeyre, 1993; Chu et al., 1993; Ragone and Burreson, 1993; Chu et al., 1996; Chu and Volety, 1997). During the late 1980s, there was a range extension into the upper reaches of Chesapeake Bay associated with four consecutive years of drought and warm winter temperatures (Burreson and Ragone-Calvo, 1996). In 1990, there was another sudden range extension into the northern side of the Delaware Bay, and from 1991 to 1995 the range extended further to its current northern limit in Maine (Ford, 1996). These extensions were postulated to have resulted from an introduction of diseased oysters to non-endemic sites, a change in either the parasite or host populations, a change in environmental conditions favorable for the parasite, or some combination thereof (Ford, 1996). The severity of disease in this new range appears to be similar to that found in the south, with decreased growth and condition index, and death in the second or third year of infection (Ford et al., 2000).

*Perkinsus marinus* occurs *in vivo* primarily as an unicellular trophozoite that develops a large vacuole which eccentrically displaces the nucleus, giving the cells a signet ring-like appearance (Perkins, 1996). In contrast with the replication solely by binary fission seen *in vitro* in ODRP-3 medium, trophozoites can multiply *in vivo* by budding or binary fission, or they can form multicellular tomonts surrounded by the parent trophozoite cell wall by palintomic fission (Fig. 1) (Perkins, 1996; Sunila et al., 2001). Tomonts may contain from 2 to 64 cells that are released by rupture of the parental cell wall (Perkins, 1996). Tomonts occur in all host tissues, but are most frequently observed intercellularly in the connective tissues, the epithelium of the gut,

gill, and digestive gland, and in hemocyte phagosomes (Perkins, 1996). Trophozoites can also differentiate into zoosporangia which, when released into seawater, undergo palintomic sporulation to yield small biflagellate zoospores that exit the zoosporangium through a discharge tube (Perkins, 1996). Trophozoites can be induced to form zoosporangia *in vitro* by incubation in thioglycollate medium. Incubation causes cell enlargement and alterations in cell wall structure which allow it to be stained with Lugol's iodine. This method is routinely used for *P. marinus* diagnosis (Ray, 1952). These enlarged trophozoites, or hypnospores, are functionally prezoosporangia, and if exposed to seawater will zoosporulate and release viable zoospores (Perkins and Menzel, 1966; Perkins and Menzel, 1967; Perkins, 1976b). For unknown reasons, however, this zoosporulation technique has failed in recent years, limiting the ability to study zoospores (Perkins, 1996). All *P. marinus* life stages are capable of initiating infection, though trophozoites may be more infective than hypnospores or zoospores (Chu, 1996; Chu and Volety, 1997). The mechanisms and regulation of differentiation into these various morphological forms are not understood, nor is it known whether the formation of zoospores represents a sexual reproductive stage.

Dermo disease is a chronic wasting syndrome that leads to slowing or cessation of growth and eventual mortality (Paynter and Burreson, 1991; Newell et al., 1994). Oysters typically acquire infections in mid- to late summer, and mortalities in the first year of growth are normally low. This is especially true in smaller oysters, presumably due to the reduced volume of water filtered and, thus, lower exposure to parasites (Burreson and Ragone-Calvo, 1996). During winter, the intensity of infection is greatly decreased, though there is evidence of a high prevalence of overwintering infections of very low intensity (Ragone-Calvo and Burreson, 1994). In the summer of the second year of infection, oyster mortality typically exceeds 90% (Paynter and Burreson, 1991; Burreson and Ragone-Calvo, 1996).



Figure 1. Life cycle of *Perkinsus marinus* during *in vitro* culture in ODRP-3 medium and during *in vivo* infection. Stage names are as described by Perkins (1996), with additional commonly used names for each stage and replicative mode listed in parentheses. The dashed line in the *in vivo* life cycle represents stages which may be external to the oyster.

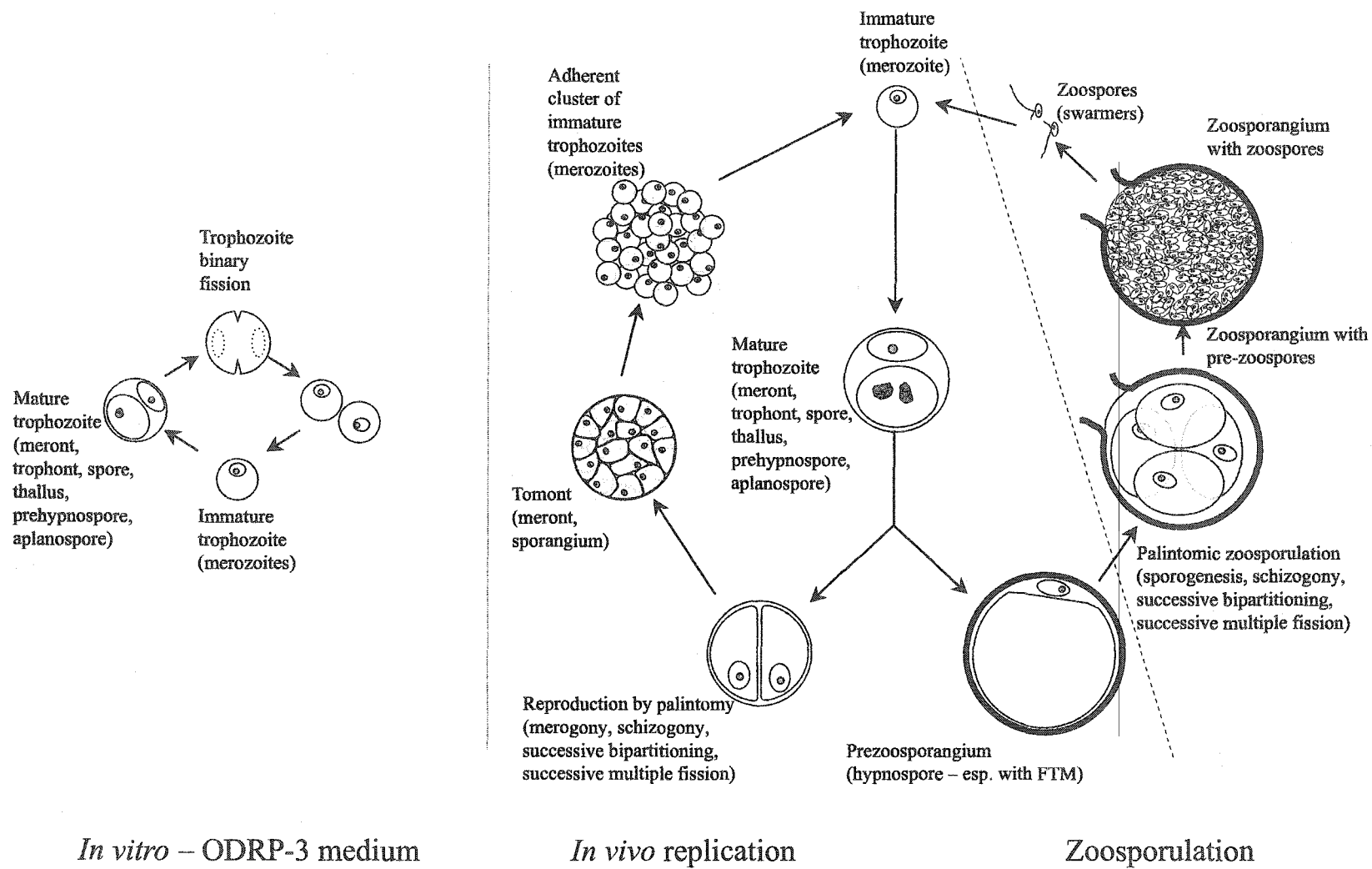


Figure 1.

Infections begin in the gill, gut, or mantle epithelium, and parasite cells are found both between host cells and within phagosomes of hemocytes. Parasite cells may be transported throughout the organism by mobile hemocytes. Alvarez (1992) demonstrated that abiotic particles introduced into the oyster digestive tract are phagocytosed and over a period of days are distributed throughout the oyster by hemocytes. In late infections, foci of *P. marinus* cells are found in all tissues. In the connective tissue and epithelium, large foci of *P. marinus* cells can cause extensive tissue lysis, abscess formation and, in heavy infections, occlusion of hemolymph sinuses (Perkins, 1976a). The exact cause of death in the oyster is not known. It is unlikely that it is due to a systemic toxin, due to the chronic nature of the disease, and the large parasite burdens that can build up prior to death (Perkins, 1976b). Death may be due to long-term energetic drain by the parasites. Oyster feeding rates and nutrient assimilation rates have been found to be identical between infected and non-infected oysters, and the calculated energy requirements of the parasite may be sufficient to account for both decreased growth rate and mortality (Choi et al., 1989; Newell et al., 1994). The metabolic costs to the oyster may also be increased by the ongoing need to repair tissue damage caused by the parasite (Choi et al., 1989).

There is evidence that *C. virginica* is able to effect limited killing of *P. marinus* cells. LaPeyre et al. (1995) found that approximately 10% of phagocytosed *P. marinus* cells viewed by electron microscopy were partially degraded 12 hours after exposure to hemocytes. The mechanism of killing is not known. It is apparent that the oyster is capable of some defensive activity toward *P. marinus*, however, it does not appear to be sufficient to effectively combat infection. This may be due to (1) the inability of extant defense mechanisms to kill *P. marinus*, (2) the parasites ability to overwhelm moderately effective oyster defenses by rapid proliferation, or (3) the ability of *P. marinus* to circumvent or downregulate relevant defense mechanisms by one or more virulence factors.

*Crassostrea virginica* defenses include both cellular and humoral mechanisms.

The hemolymph is clear, devoid of respiratory pigments, and contains hemocytes within a complex mixture of plasma proteins. These are distributed throughout the oyster by an open circulatory system that conveys hemolymph through sinuses and intracellular spaces in direct contact with tissue cells (Ebel and Scro, 1996). Hemolymph contains three types of cells, the granulocytes, hyalinocytes, and the serous, or brown, cells.

Granulocytes account for the majority of circulating hemocytes and contain a mixture of acidophilic, basophilic and refractile granules and form filopodial extensions when adhered to surfaces (Foley and Cheng, 1972; Auffret, 1988; Cheng, 1996). Hyalinocytes contain few to no granules and tend to form broad cytoplasmic extensions, or lobopodia (Foley and Cheng, 1972; Cheng, 1996). Serous cells are found predominantly in the excretory Keber's glands in the atria and mantle and, though they are capable of migration, are not considered to be true hemocytes (Cheng, 1996). They are believed to play a role in removal of products of parasite metabolism and degradation (Cheng, 1996).

The primary defensive role of hemocytes is phagocytosis and intracellular degradation of pathogens, though they also appear to be responsible for production of many of the humoral defense molecules (Cheng and Roderick, 1975; Cheng, 1976; Mohandas and Cheng, 1985; Mohandas et al., 1985; Chu, 1988; LaPeyre, Chu and Meyers, 1995; LaPeyre, Chu and Vogelbein, 1995; Cheng, 1996). *In vitro*, granulocytes are more actively phagocytic toward both bacteria and *P. marinus* than are hyalinocytes (Foley and Cheng, 1975; LaPeyre, Chu and Meyers, 1995; LaPeyre, Chu and Vogelbein, 1995). Oyster hemocytes chemotactically respond to Gram positive and negative bacteria, as well as toward *P. marinus* cells and cell lysate (Cheng and Howland, 1979; Howland and Cheng, 1982; Garreis et al., 1996). The receptor or receptors driving these chemotactic responses are not known.

Phagocytosis requires initial surface attachment of the particle to the hemocyte, and thus some ability to detect and discriminate self from non-self particles. Some non-

self recognition is based on physical properties of both the hemocyte and the particle (e.g. hydrophobicity, charge); however, there is evidence that there is recognition in invertebrates which goes beyond simple detection of bulk physical traits (Anderson, 1975; Bigger, 1984; Cheng, 1987). Self/non-self discrimination and pre-phagocytic binding in the oyster may be based on carbohydrate recognition by lectins, which are glycoproteins that specifically bind the carbohydrate moieties of other glycoproteins (Sminia and van der Knapp, 1986). Invertebrate lectins may be integral in the hemocyte membrane, free in hemolymph, bound to cell-surface lectin receptors, or stored within hemocytes (Yeaton, 1981b; Vasta et al., 1982; Vasta et al., 1984; Sminia and van der Knapp, 1985; Vasta and Marchalonis, 1985; Arizza et al., 1991). They can exist singly or as non-covalently bound multimers of single or combined specificities and can function as opsonins or, in their multivalent form, as agglutinins (Yeaton, 1981a; Chu, 1988; Fisher and DiNuzzo, 1991; Kawabata and Iwanaga, 1999). Integral membrane lectins may be capable of signal transduction upon binding their ligand (Kwaik et al., 1998). Thus, lectins may play a major role in both cellular and humoral defenses in the oyster by providing a potential mechanism for self/non-self recognition and cellular activation, and through their functions as agglutinins and opsonins.

*Crassostrea virginica* has at least two serum lectins of differing carbohydrate specificity, as well as a membrane-bound lectin on hemocytes which bears the same specificity as one of the serum lectins (McDade and Tripp, 1967b; Vasta et al., 1982; Vasta et al., 1984). Fisher and DiNuzzo (1991) tested the agglutinating capability of oyster serum against 94 bacterial isolates as well as red blood cells from seven vertebrate species and found that oyster serum agglutinated all red blood cell types, as well as 64 of the 94 bacterial isolates tested. There was some specificity in the agglutination reaction, with variations in agglutination intensity between different isolates of the same species, for example all 01-serotype *Vibrio cholerae* were agglutinated, while non-01-serotype were not (Fisher and DiNuzzo, 1991). The agglutinating molecule or molecules were

heat labile, but were not isolated nor further characterized; the assumption was made that they were multivalent lectins.

The role of agglutinating or opsonic lectins in *P. marinus* infection is not known. Chintala et al. (1994) found no correlation between parasite density or survival time and serum agglutination titer to *Vibrio cholerae* or horse or human red blood cells. Given the possibility of involvement of lectins of differing specificity, however, the role of lectins in *P. marinus* defense cannot be excluded. The exact mechanisms by which *C. virginica* hemocytes distinguish pathogens, including *P. marinus*, have not been elucidated. While oyster hemocytes clearly recognize and phagocytose *P. marinus* cells, the occurrence of up to half of infecting *P. marinus* cells free in tissues and hemolymph suggests a masking of the parasite from recognition by oyster hemocytes (Cheng and Dougherty, 1994), or inactivation of hemocyte phagocytic function.

Following phagocytosis, *C. virginica* hemocytes are capable of both oxygen-dependent and oxygen-independent killing and degradative mechanisms within phagosomes (Anderson, 1996). The initiation of oxygen-dependent killing mechanisms, the respiratory burst, is marked by a rapid use of oxygen in creation of toxic reactive oxygen species (ROS) (Anderson, 1994). The progenitor of all ROS is the superoxide anion ( $O_2^-$ ), which is produced by the univalent reduction of molecular oxygen by a membrane-bound NADPH-oxidase. The superoxide anion may then spontaneously dismute, or be dismuted by the enzyme superoxide dismutase (SOD), to hydrogen peroxide. Further enzymatic processing of hydrogen peroxide may yield other reactive species such as hydroxyl radicals, singlet oxygen and, in the presence of myeloperoxidase enzyme and halide species, hypohalous acids (Klebanoff, 1968; Pipe, 1992; Anderson et al., 1995). Torreilles and Guerin (1999) have documented production of another highly toxic ROS product, peroxynitrite in the hemocytes of the mussel *Mytilus galloprovincialis*. Using a variety of chemiluminescent probes (e.g. lucigenin for superoxide anion; luminol for myeloperoxidase activity) and ROS inhibitors and

scavengers (e.g. superoxide dismutase, catalase, taurine, mannitol, azide), Austin and Paynter (1995) showed that hemocytes of *C. virginica* are capable of producing superoxide anion, hypochlorous acid (HOCl), and probably hydrogen peroxide in response to phagocytosis of zymosan, a particulate yeast cell wall preparation, or treatment with the protein kinase C activator, phorbol myristate acetate. They also found a positive relationship between the level of ROS production on exposure to zymosan or PMA and the underlying *P. marinus* infection intensity. Anderson et al. (1995) similarly found that zymosan-induced chemiluminescence was higher in oysters with moderate to heavy *P. marinus* infections. Phagocytosis of *P. marinus* trophozoites does not, however, induce a chemiluminescent response in oyster hemocytes, implying a priming effect on ROS production coupled with non-activation or suppression of activation of actual ROS generation, or a scavenging of ROS (Volety and Chu, 1995; Anderson, 1999). Bramble and Anderson (1999) question the significance of the ROS response in oyster defense based on a low level of response, in comparison with striped bass phagocytes, to challenge with *Bacillus megaterium* or *Pseudomonas fluorescens*.

Volety and Chu (1995) found that the luminol-enhanced chemiluminescence measurement of zymosan-induced ROS response was suppressed by introduction of live, but not heat-killed, *P. marinus* trophozoites. Anderson (1999) demonstrated the same effect using live and osmotically killed *P. marinus*. He further quantified the inhibition using lucigenin to specifically detect superoxide anion. These results indicate a possible up-regulation or release of inhibitory or scavenging molecules in the extracellular products (ECP) of *P. marinus* upon phagocytosis. Scavenging of ROS by molluscan parasites has been demonstrated in the gastropod mollusc *Biomphalaria glabrata*. The sporocyst of *Schistosoma mansoni* secretes an antioxidant molecule which suppresses the effectiveness of the hemocyte ROS response in the snail (Connors and Yoshino, 1990; Connors et al., 1991). In humans, a phosphatase secreted by *Leishmania* blocks superoxide anion production in neutrophils (Remaley et al., 1984).

The oxygen-independent cellular defense mechanisms in the oyster overlap significantly with many of the humoral defense mechanisms. The presence of several lysosomal enzymes, including acid and alkaline phosphatases,  $\beta$ -glucuronidase,  $\alpha$ -mannosidase, lipase, and lysozyme occurs both in hemocytes as well as free in oyster serum (Roderick and Cheng, 1974; Cheng and Roderick, 1975; Cheng, 1976; Chu and LaPeyre, 1989; Cheng, 1992; LaPeyre, Chu and Meyers, 1995). Fusion of cytoplasmic granules with the phagosomes releases lysosomal enzymes which can kill and degrade pathogens phagocytosed by the cell. There is evidence that hemocyte granules may also be the source of some components of molluscan humoral immunity. Lysosomal enzymes contained in granules in the hemocytes of *Mercenaria mercenaria*, for example, are released into the serum within a dual-membrane vesicle that degrades in the serum (Mohandas and Cheng, 1985; Mohandas et al., 1985). Oysters also show inducible release of lysosomal enzymes into the serum. Cheng (1992) measured the serum levels of two lysosomal enzymes, acid phosphatases and  $\alpha$ -mannosidase following exposure to four bacterial species. Oyster hemocytes apparently selectively released lysosomal enzymes to the serum upon exposure to *Escherichia coli* and *Salmonella* sp., but not on exposure to *Klebsiella oxytoca* or *Micrococcus roseus* (Cheng, 1992). The association of enzyme release with hemocyte recognition and/or phagocytosis of the bacteria was not addressed. Lysozyme, a lytic lysosomal enzyme capable of catalyzing the cleavage of the  $\beta$ -1,4 linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial cell walls, has been extensively studied as a mechanism of humoral defense in *C. virginica* (McDade and Tripp, 1967a; Roderick and Cheng, 1974; Chu and LaPeyre, 1989; LaPeyre, Chu and Meyers, 1995). While oyster lysozyme is effective against numerous Gram positive and Gram negative bacterial isolates, it does not appear to play a role in *Perkinsus* defense (Chu and LaPeyre, 1989).

In addition to the agglutinating and lysosomal proteins described above, oysters have humoral defense mechanisms mediated by low molecular weight proteins. Oysters



have been reported to have a <10 kDa molecule active against *P. marinus*, however, it was of very limited effect *in vitro*, and is of questionable significance (Anderson and Beaven, 2001). Oysters also have a peptide (< 10 kDa) serum component that acts as a protease inhibitor (Oliver et al., 1999). The level of protease inhibition in the serum was positively correlated with the susceptibility of oyster lines selectively bred for decreased *P. marinus* susceptibility (Oliver et al., 2000).

The particular molecular determinants of virulence in *P. marinus* are not known, though there is growing evidence that proteolytic enzymes in the ECP of *P. marinus* may play an important role in parasite virulence and modulation of host defenses (Garreis et al., 1996; LaPeyre et al., 1996; Oliver et al., 1999). Proteases have been shown to be virulence factors in several protozoan pathogens (McKerrow et al., 1993). Pathogenicity is associated with total protease production in the apicomplexan parasite *Babesia bovis* (Wright et al., 1981). While still infective, strains that do not produce protease fail to cause the fatal disease normally associated with infection (Wright et al., 1981). The cercarial proteases of schistosomes play a direct role in parasite virulence; their inhibition, while not interfering with parasite motility or attachment processes, prevents skin penetration (McKerrow and Doenhoff, 1988; McKerrow et al., 1993). Amastigote and promastigote forms of *Leishmania mexicana* secrete cysteine proteases crucial to their survival within phagosomes of mammalian macrophages (Coombs, 1982; Pupkis and Coombs, 1984). One leishmanial protease, leishmanolysin or gp63, is a virulence factor involved in host macrophage binding and complement inactivation; its upregulation leads to higher infectivity of the parasite (Ramamoorthy et al., 1992; McKerrow et al., 1993; McGwire et al., 2002). *Trypanosoma cruzi*, the causative agent of Chagas' disease, secretes the cysteine protease cruzipain which is required for parasite replication and differentiation (McKerrow et al., 1993). In the *Plasmodium* malarial parasites, sets of proteases variously involved in erythrocyte penetration, hemoglobin digestion, and erythrocyte rupture are differentially expressed during trophozoite,

schizont and merozoite life stages (Rosenthal et al., 1987; Braun-Breton and Pereira da Silva, 1988; McKerrow et al., 1993). Other protozoan parasites, including *Trypanosoma brucei*, *Trichomonas vaginalis*, and *Giardia lamblia*, secrete proteases which are of unknown significance in virulence (McKerrow et al., 1993).

The study of protease secretion by *P. marinus* began with assessment of relative amounts of protease contained within ECP by casein hydrolysis (LaPeyre and Faisal, 1995b). The proteases were further investigated and categorized as serine proteases based on protease inhibitor studies (LaPeyre, Schafhauser et al., 1995). One or more constituents of *P. marinus* ECP appears to be involved in virulence, as oysters fed *P. marinus* ECP encapsulated in liposomes had a sevenfold increase in *P. marinus* body burden after experimental infection (LaPeyre et al., 1996). The mechanism behind this increase is not known, but may be due to a protease-mediated disruption of the gut epithelium favoring parasite invasion, increased availability of nutrients leading to increased parasite replication, or a disruption of oyster defense mechanisms. Oliver et al. (1999) demonstrated that a 35 kilodalton *C. virginica* hemolymph protein of unknown function was degraded in the presence of ECP. Subsequent studies have demonstrated that nearly all oyster hemolymph proteins are susceptible to degradation by *P. marinus* ECP proteases (Earnhart, unpublished results). Only limited attempts have been made to isolate and purify individual proteases for structural investigation, and there have been no experiments designed to elucidate specific functional roles for these proteases. The DNA sequence of one *P. marinus* protease has been determined, and bears sequence homology with the subtilisin class of serine proteases; however, the function of the protease is not known, nor is it clear if this protease is secreted extracellularly (Brown, 2001). There currently exists no adequate means of quantification or functional analysis of individual proteases either *in vitro* or *in vivo*, nor have there been genetic analyses specifically targeting the secreted proteases for assessment of their classification and relatedness to known virulence factors.

The study of the excretory products and, in particular, proteases of *P. marinus* has been greatly facilitated by the development of methods of *in vitro* culture. There are several media for *in vitro* *P. marinus* culture, ranging from a chemically defined protein-free medium to media containing such supplements as yeastolate, cod liver oil, bovine serum albumin, fetal bovine serum (FBS), and fetuin (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; LaPeyre et al., 1993; Dungan and Hamilton, 1995; Gauthier et al., 1995; LaPeyre and Faisal, 1997). The effects of supplementing *P. marinus* media with oyster tissue homogenates and hemolymph from both *P. marinus*-susceptible and tolerant oyster species have also been studied. This supplemented media produces marked changes in cell proliferation as well as morphology and differentiation (Gauthier and Vasta, 2002; MacIntyre et al., 2003). In addition, there are significant changes in the pattern of protease expression when *P. marinus* cells are exposed to *C. virginica* homogenates. Those changes are not seen when cells are exposed to homogenates from the more tolerant oyster species *C. gigas* and *C. ariakensis* (MacIntyre et al., 2003). The ECP of cells grown in chemically defined medium displays only the apparently constitutively-expressed higher molecular weight proteases. Supplementation of this media with oyster products causes dose-responsive changes in intensity of proteolytic bands, and alteration in protease profile including both apparent downregulation of high molecular weight, and upregulation of low molecular weight proteases (MacIntyre et al., 2003).

In order to address their significance in *P. marinus* virulence, proteases have been purified as a class from the ECP of *P. marinus* cells grown in defined and supplemented media using affinity chromatography. This purification technique relies on the affinity of a protease active site for either a substrate or for a specific or non-specific reversible enzyme inhibitor. Proteases, including *P. marinus* serine proteases, can be purified using the cyclopeptide antibiotic bacitracin (van Noort et al., 1991; Faisal et al., 1999). Both whole ECP and purified ECP proteases have been shown to affect several important

defense parameters *in vitro*. The normal chemotactic response of oyster hemocytes across a microporous filter toward *P. marinus* cells or *P. marinus* cell lysate was significantly reduced when *P. marinus* ECP or bacitracin-purified protease was co-located with the cells or lysate (Garreis et al., 1996). *Perkinsus marinus* ECP and purified protease also decreased the level of random, non-stimulated migration across the a microporous filter (Garreis et al., 1996). Coincubation of *C. virginica* serum with ECP also decreased the level of lysozyme activity and reduced the titer of agglutinins toward sheep red blood cells (Garreis et al., 1996). As has been discussed previously, *P. marinus* ECP may also contain products that scavenge or downregulate production of ROS upon phagocytosis by oyster hemocytes (Volety and Chu, 1995; Anderson, 1999).

Assessment of induction, expression levels, elaboration, and function of possible virulence factors in the ECP of *P. marinus* requires specific and sensitive methods of detection and purification. Attempts have been made to create antibodies against both *P. marinus* whole cells and ECP. Choi et al. (1991) produced a rabbit polyclonal antiserum to lysed hypnospores created by incubation of infected oyster tissues in fluid thioglycollate medium. While hypnospore protein could be sensitively detected both by enzyme-linked immunosorbant assay (ELISA) and immunofluorescent staining of whole hypnospores, the antiserum failed to react with any other form of the parasite (Choi et al., 1991). There was also significant crossreactivity of the antiserum with uninfected oyster tissue, requiring adsorption of crossreactive antibody with acetone-dried oyster tissue and oyster egg powder. This crossreactivity was attributed to either antigenic similarity between oyster and *P. marinus* proteins, or to embedded oyster cell membranes within the hypnospore wall (Choi et al., 1991). While not explicitly stated, the immunization regimen utilized in that study implied that the hypnospores may have been poorly immunogenic, as the initial immunization of 1 mg of protein was followed by five 0.5 mg immunizations prior to antiserum collection.

Dungan and Roberson (1993) created rabbit polyclonal antibodies using four injections of  $3.0 \times 10^6$  paraformaldehyde-fixed whole hypnospores created in fluid thioglycollate media. By ELISA, there was some minor crossreactivity with oyster proteins. The polyclonal antiserum recognized all life stages of *P. marinus* and was successfully used for fluorescent immunostain detection of *P. marinus* cells in tissue section. Reactive cells were clearly visible in infected epithelial and connective tissues, as well as within the lumen of the digestive tract. The tissues surrounding the *P. marinus* cells displayed a diffuse fluorescence that may have been due to secreted ECP detected by the polyclonal antiserum. The antibodies crossreacted with other members of the genus *Perkinsus* as well as other dinoflagellate species (Dungan and Roberson, 1993; Dungan et al., 2000). Bushek et al. (2002) tested the reactivity of this polyclonal antiserum against nineteen free-living and eight parasitic dinoflagellates. They found reactivity with seven of the parasitic dinoflagellates and three of the free-living species. Polyclonal antiserum against one of the reactive parasitic dinoflagellates, *Hematodinium* sp., also crossreacted with those same parasitic dinoflagellates and with *P. marinus*, indicating shared epitopes among these species. When crossreactive binding occurred, it was not confined to surface determinants, but was often found in the cytoplasm and nucleus as well. The identity of the crossreactive immunogen or immunogens is not known.

Monoclonal antibody production was also attempted by Dungan and Roberson (1993) using mice immunized with hypnospores of *P. marinus*. Mice initially immunized with  $10^5$  cells in Freund's complete adjuvant (CFA), then boosted twice with  $1.0$  to  $3.0 \times 10^5$  cells failed to produce an adequate serum titer. Only after repeated subcutaneous immunizations with and without CFA was the titer satisfactory to allow fusion. Of the 26 ELISA-positive hybridomas created, most were of the IgM isotype, and none bound *in vivo* cells in immunostaining assays.

Monoclonal antibodies to whole *P. marinus* cells were produced by Romestand, et al. (2001) by three immunizations of  $3 \times 10^6$  previously frozen trophozoites. Two hybridomas were selected and were incorporated into a competitive ELISA format capable of measuring proliferation of *P. marinus* in *in vitro* culture. The antibodies bound to all life stages of *P. marinus*, but were cross-reactive with *P. atlanticus* (now synonymized with *P. olsenii* (Murrell et al., 2002)).

Montes et al. (2002) prepared a polyclonal rabbit antiserum against several proteins from lysed hypnospores of *Perkinsus atlanticus* which had been excised from a preparative SDS-PAGE gel. An antiserum recognizing a 233 kDa protein was selected for further study and that protein was found to be a major cell wall component of *P. atlanticus*. The polyclonal antiserum bound to all walled life stages, and was cross-reactive on immunoblot with a *P. marinus* protein of similar size.

Ottinger et al. (2001) used the ECP of *P. marinus* grown in a chemically defined medium to create polyclonal rabbit antiserum. This antiserum was used for comparison of *in vitro* with *in vivo* ECP protein secretion, for development of a quantitative ELISA for measurement of ECP production, and to attempt correlation of *in vivo* ECP levels with infection intensity. Immunoblots of negative, lightly, and heavily infected oysters were used to assess binding of the antiserum to putative *in vivo* components of ECP. Seventeen *in vitro*-produced ECP proteins were recognized by the antiserum by immunoblot. Three of these were identical to those found in infected oysters. There were two additional proteins detected that did not correspond to any *in vitro* produced protein.

The intensity of infection, as assessed by ELISA, was in disagreement with the fluid thioglycollate assay for *P. marinus* infection from 40% to 45% of the time. The majority of the disagreement was due to ELISA-positive, FTM-negative findings, which could have been caused by the greater sensitivity of the ELISA assay. There was a blocking effect of oyster homogenate, and a low level of crossreactivity with oyster

tissues that hampered detection of low ECP concentrations. During attempts to reproduce and expand on the findings of Ottinger et al. (2001), new batches of polyclonal rabbit antisera were produced, which proved to be crossreactive with non-infected oyster tissue and hampered efforts to reproduce the ELISA and immunoblot results. Furthermore, the anti-ECP titer in each of two identically treated rabbits was low, despite repeated boosts (Earnhart, unpublished results).

Given the magnitude of the decline in oyster populations due to *P. marinus*, there is a critical need for directed research into the specific mechanisms by which the differentiation and virulence of this parasite are regulated. Recently, Ford et al. (2002) confirmed a multitude of anecdotal evidence regarding the rapid loss of parasite virulence during *in vitro* culture. This may provide a useful tool for the study of critical virulence factors, especially if a reliable mode of restitution of virulence for *in vitro* cultures can be uncovered. Evidence to date suggests that ECP, and especially the ECP proteases, may play a significant role in virulence. Despite a number of technical hurdles to be overcome, creation of antibodies specific to ECP components will allow a needed understanding of their regulation and role in virulence.

## Chapter 2

### Supplementation of *Perkinsus marinus* cultures with host plasma or tissue homogenate enhances their infectivity

#### Abstract

The protozoan oyster parasite, *Perkinsus marinus*, can be cultured *in vitro* in a variety of media; however, this has been associated with a rapid attenuation of infectivity. Supplementation of defined media with products of *P. marinus*-susceptible (*Crassostrea virginica*) and tolerant (*C. gigas*, *C. ariakensis*) oysters alters proliferation and protease expression profiles, and induces differentiation into morphological forms typically seen *in vivo*. It was not known if attenuation could be reversed by host extract supplementation. To investigate correlations among these changes as well as their association with infectivity, the effects of medium supplementation with tissue homogenates from both susceptible and tolerant oyster species were examined. The supplements markedly alter both cell size and proliferation, regardless of species; however, upregulation of low molecular weight protease expression is most prominent with susceptible oysters extracts. Increased infectivity occurs with the use of oyster product-supplemented media, but is not consistently associated with changes in cell size, cell morphology, or protease secretion, and is not related to the susceptibility of the oyster species used as the supplement source.



## Introduction

*Perkinsus marinus* is a protozoan parasite of the eastern oyster, *Crassostrea virginica*. Mortalities caused by this parasite typically occur in the second summer of infection, and have been responsible for much of the recent decline in the oyster fishery along the eastern seaboard of the United States (Bureson and Ragone-Calvo, 1996; Ford, 1996). The molecular mechanisms of parasite infectivity, virulence, and interaction with the host defense system are largely unknown. The development of media formulations allowing axenic culture of *Perkinsus marinus* has provided new opportunities to assess the effects of host components on parasite growth, physiology, and infectivity. There are several media formulations for *in vitro* *P. marinus* culture that employ commercial base formulations (e.g. Dulbecco modified Eagle's medium with Ham's F12 nutrient mixture) supplemented with such constituents as cod liver oil, bovine serum albumin, yeastolate, fetal bovine serum (FBS), or its  $\alpha$ -fetoprotein constituent, fetuin (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; LaPeyre et al., 1993; Dungan and Hamilton, 1995; Gauthier et al., 1995; Gauthier and Vasta, 1995). There is also a chemically defined, protein-free medium (ODRP-3; LaPeyre and Faisal, 1997) which has proven to be of particular value for the production of antibodies against *P. marinus* extracellular products (Earnhart and Kaattari, 2003).

The *in vivo* *P. marinus* life cycle begins with a small, immature trophozoite, that enlarges over time into a "signet ring" form, so named for its large vacuole and offset nucleus. This mature trophozoite may then undergo palintomic fission in which 4 to 64 or more immature trophozoites are formed within, then exit from, the parental cell, or tomont, wall (Perkins, 1996). *Perkinsus marinus* can also form motile zoospores, again by palintomic fission, with exit of the zoospores through a discharge tube and pore structure formed on the wall of the enlarged parental trophozoite, the zoosporangium (Perkins, 1996). During *in vitro* culture in ODRP-3 medium, cellular proliferation is apparently solely by binary fission, and no zoosporulation is seen. In an effort to more

closely simulate, *in vitro*, the milieu to which *P. marinus* is exposed *in vivo*, oyster tissue homogenate and plasma from *P. marinus*-susceptible and tolerant oyster species have been employed as culture supplements (Gauthier and Vasta, 1995; Gauthier and Vasta, 2002; MacIntyre et al., 2003). Compared with growth in unsupplemented ODRP-3, *Perkinsus marinus* cells grown in the presence of plasma supplements from *Crassostrea gigas*, *Crassostrea ariakensis*, or infected *C. virginica* oysters show reduced *in vitro* proliferation (Gauthier and Vasta, 2002). Uninfected *C. virginica* plasma supplementation, however, results in only minimal inhibition of proliferation (Gauthier and Vasta, 2002; MacIntyre et al., 2003). Oyster tissue homogenate supplemented media produces marked changes in cell proliferation, morphology, and differentiation, including enlargement of trophozoites and induction of tomont stages, which are rarely seen in unsupplemented ODRP-3 medium, but commonly observed during infection (MacIntyre et al., 2003).

Several studies have implicated proteases as contributing factors in *P. marinus* virulence (Garreis et al., 1996; LaPeyre et al., 1996; Oliver et al., 1999; Oliver et al., 2000). The extracellular products of *P. marinus* cells grown in ODRP-3 contain only high molecular weight (>50 kDa) proteases (MacIntyre et al., 2003). However, when *C. virginica* plasma or homogenate is used as a media supplement, there is significant alteration in *P. marinus* protease expression patterns, including a simultaneous down-regulation of high molecular weight, and upregulation of low molecular weight (<50 kDa) proteases. These changes are not seen when cells are exposed to homogenates from *C. gigas* and *C. ariakensis* oysters (MacIntyre et al., 2003), both of which have been reported to be more tolerant of *P. marinus* infection than is *C. virginica* (Meyers et al., 1991; Barber and Mann, 1994; Calvo et al., 2000; Calvo et al., 2001).

It is not clear if there is an association between the observed changes in cellular morphology and the shifts in the secreted protease profiles in supplemented cultures, or whether either is associated with parasite infectivity. In order to better understand these

relationships, protease production, *in vitro* cell size and morphology, and infectivity were assessed under various conditions. Observations were made of variations in these factors among clonal *P. marinus* isolates, then one isolate was selected for investigation of alterations in the presence of various host-derived media supplements. A detailed observation of the effects of tissue-based supplements derived from susceptible and tolerant oysters was then assessed, and a selected dosage level was used to investigate infectivity using a variety of oyster populations and species.

## Materials and methods

### *Animals*

Experimental oysters were maintained in 1µm filtered York River (Virginia, USA) water and fed commercially produced algae (Reed Mariculture, San Jose, CA). Water changes were performed twice weekly, and all effluent water was chlorinated prior to release. *Perkinsus marinus*-free *C. virginica* oysters from Maine (*C. virginica* ME; Pemaquid Oyster Company, Waldoboro, ME) were initially employed, but the occurrence of *P. marinus* infections in those oysters over the course of this study necessitated the importation of disease-free *C. virginica* oysters from Washington state (*C. virginica* WA; Taylor Shellfish Farms, Shelton, WA). *Crassostrea gigas* oysters were purchased from Taylor Shellfish Farms. *Crassostrea virginica* oysters originally from populations in Louisiana (*C. virginica* LA), Tangier Sound (*C. virginica* TG) in the Chesapeake Bay, Virginia, and the CROSBreed program (*C. virginica* XB; selectively bred for *P. marinus* resistance) were all gathered from a single deployment site in the Yeocomico River, a tributary of the Potomac River (Virginia, USA) near its entrance to the Chesapeake Bay. *Crassostrea ariakensis* oysters were provided by the Virginia Institute of Marine Science oyster hatchery. A subsample of both the *C. virginica* ME and *C. virginica* WA oysters used in infection trials was confirmed to be *P. marinus*-free by body burden analysis (see below).

### *Infection trials and analysis of *P. marinus* body burden*

Oysters were infected by injection of parasites into the mantle cavity through a small hole in the shell made with a lapidary saw. Each oyster was injected with  $10^6$  parasite cells suspended in 100  $\mu$ L of artificial sea water (20 ppt; Forty Fathoms Marine Mix, Marine Enterprises International, Baltimore, MD) on days 1, 3 and 5, and a sham group was injected with artificial sea water. The oysters were maintained at a density of ten to fifteen per 35 L aquarium and sacrificed after four weeks for analysis of *P. marinus* infection level.

In order to most accurately and sensitively quantify the infection level following experimental infection, *P. marinus* cells were enumerated using the whole body burden technique (Choi et al., 1989; Fisher and Oliver, 1996). Briefly, the oyster soft tissues were finely minced and incubated at room temperature for seven days in 20 mL fluid thioglycollate medium (2.9% FTM, T-9032, Sigma-Aldrich, Inc., St. Louis, MO; 2% NaCl) supplemented with penicillin/streptomycin solution (5% (v/v); P-0781, Sigma-Aldrich, Inc.). Following incubation, the tissue was pelleted by centrifugation, resuspended in 20 mL 2M NaOH, and held at 60°C for three to four hours to dissolve the oyster tissue. The enlarged *P. marinus* prezoosporangia (hypnospores) were then pelleted, washed three times in distilled water, stained with a 1:5 dilution of Lugol's iodine, immobilized on a 0.45  $\mu$ m filter by vacuum, and enumerated by light microscopy. When necessary, dilutions of the hypnospores were made in distilled water, counted in triplicate, and the mean count extrapolated to the volume of the sample. The resultant counts were  $\log_e$  transformed for analysis of variance and for Tukey's multiple comparisons test, using a 5% error rate.

### *Analysis of proteolytic enzyme expression*

Supernatants from *P. marinus* cultures and their respective media controls were analyzed for protease production by zymography. Twenty  $\mu$ L of media or culture

supernatant were electrophoresed under non-reducing conditions by SDS-PAGE (Laemmli, 1970) using an 8% polyacrylamide separating gel copolymerized with 0.1% porcine gelatin (G-8150, Sigma-Aldrich, Inc.), and a 4% stacking gel. Following electrophoresis, the gels were washed three times for ten minutes in 2.5% Triton-X-100 (BP151, Fisher Scientific, Fairlawn, NJ) to renature the proteins, then once in 0.1 M Tris HCl (BP152, Fisher Scientific), pH 8.0. The gels were then incubated at 37°C in a fresh change of Tris HCl, pH 8.0 buffer. While use of buffers from pH 4.0 to pH 9.0 have previously been shown not to alter the *P. marinus* zymographic profile, pH 8.0 is optimum for detection of *P. marinus* proteolytic activities by zymogram (LaPeyre et al., 1995), and has been specifically used for the study of the modulation of protease expression under varying media supplementation schemes (MacIntyre et al., 2003). Following incubation, the gels were stained overnight in Coomassie brilliant blue G-250 (0.1%; 161-0406, Biorad, Hercules, CA) in 40% methanol, 10% acetic acid. The gels were destained in the same solution, without the stain. The gels were then assessed for the presence of cleared bands corresponding to the location of gelatinolytic proteases. Since the gels were, by necessity, run under non-reducing conditions, no attempt was made to assign absolute molecular weights to them. The proteases are, for convenience, referred to as high (>50kDa) and low (<50kDa) molecular weight species.

#### *Assessment of differences in infectivity among several isolates of P. marinus*

*Perkinsus marinus* clonal isolates LA10-1 (ATCC#50906; genotype #4), MA2-11 (ATCC#50896; genotype #1), SC3-2 (genotype #3) (Bushek et al., 2000), and P-1 (genotype #9) (Faisal et al., 1999), and the isolate HVA-18 (ATCC#50764; genotype #8) (Bushek et al., 2000) were cultured for use in an infection trial, and were selected based on previous genotype identification at eight polymorphic loci (Reece et al., 2001). The HVA-18 isolate was not initially cloned; however, genetic analysis of this isolate at three loci showed little genetic variability, supporting the possibility that it has become clonal

by serial passage (unpublished data). All cultures were seeded at  $10^6$  cells per mL in 75 cm<sup>2</sup> flasks containing 50 mL of a supplemented DMEM-Ham's medium with 5% fetal bovine serum commonly known as "Dungan's medium" (Dungan and Hamilton, 1995; Ford et al., 2002). Cultures were maintained in a humidified incubator at 27°C under 5% CO<sub>2</sub> for three weeks, harvested for oyster challenge, and the culture supernatants assayed by zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size was estimated using an ocular micrometer. Twenty *C. virginica* ME oysters per group were subjected to experimental infection.

*Assessment of the effects of oyster plasma and tissue homogenate supplementation on P. marinus infectivity*

The P-1 isolate of *P. marinus* was chosen for further study based on its demonstrated potential to alter its secretion profile of high and low molecular weight proteases following media supplementation. Both plasma and tissue homogenate supplemented media induce similar changes in P-1 protease expression, but only homogenate alters cell size and morphology, allowing the opportunity to distinguish correlations between these alterations and parasite infectivity. Hemolymph was withdrawn from the adductor muscle through a shell notch in three *C. virginica* ME oysters, using a syringe fitted with a 25 gauge needle. The hemocytes were removed by centrifugation ( $500 \times g$ , 5 minutes), and the plasma was pooled and filtered through a 0.22  $\mu m$  filter ( $\mu$ Star LB, Costar, Corning, Inc., Acton, MA). The oyster tissues were pooled and processed for homogenate basically in the manner described in MacIntyre et al. (2003). Briefly, the oyster soft tissue was finely minced, suspended in 10 mL of cold artificial sea water, and homogenized using a glass Tenbroek homogenizer. This homogenate was centrifuged for 30 minutes at  $2,500 \times g$  at 4°C, and the supernatant withdrawn and centrifuged at  $12,000 \times g$  for an additional 30 minutes at 4°C. This

partially clarified homogenate was then filtered to 0.22  $\mu\text{m}$  by syringe filters, and the protein content assayed by the bicinchoninic acid technique (BCA, Pierce, Rockford, IL).

Cultures of the P-1 isolate of *P. marinus* were seeded at  $10^6$  cells per mL in 75  $\text{cm}^2$  flasks containing 50 mL of ODRP-3 media without supplementation, or supplemented with 0.3 mg/mL of plasma or 0.3 mg/mL of tissue homogenate. The *P. marinus* cells used in this experiment had been cultured on a long term basis in unsupplemented ODRP-3 prior to exposure to the media supplements. Cultures were maintained in a humidified incubator at 27°C under 5%  $\text{CO}_2$  for four weeks. Cells were then harvested for oyster challenge, and the culture supernatants assayed by zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size estimated using an ocular micrometer. Thirty *C. virginica* ME oysters per group were subjected to experimental infection.

*Assessment of the effects of homogenate supplementation on P. marinus cell size, morphology, and life stages*

In view of the restitution of infectivity and superior induction of proteases by oyster homogenate (see results below), it was elected to investigate in greater detail the effects of homogenate supplementation on the *P. marinus* P-1 isolate. Cells were cultured in ODRP-3 medium supplemented with homogenates of tissues from five individual *C. virginica* WA, *C. gigas*, and *C. ariakensis* oysters. Fetal bovine serum (FBS) and fetuin were used as control supplements. ODRP-3 medium was supplemented at 1.0, 0.33, 0.11, 0.037, 0.012, and 0.004 mg/mL on a protein basis, or was left unsupplemented. Each well was seeded with  $10^6$  *P. marinus* cells in one mL of media in duplicated 48-well tissue culture plates. An additional set of unseeded plates were maintained as media controls. All plates were held in a humidified incubator at 27°C under 5%  $\text{CO}_2$  for six weeks.

Following incubation, the cells were resuspended ( $30 \times 100 \mu\text{L}$  passages through a  $200 \mu\text{L}$  pipette tip) prior to hemacytometer counts of single and clustered trophozoites. The exact enumeration of the cells within clusters was not possible, as they were extremely adherent, thus clusters were ranked as groups of two to three, four, five to 16, and greater than 16 cells. Additionally, measurement of cell diameters of ten trophozoites were completed for each culture well using an ocular micrometer, at a magnification of 400X. In order to simultaneously compare changes in both cell volume and count, a “pellet volume” was calculated as the average cell volume ( $((4/3)\pi r^3)$ ) multiplied by the estimated total cell number, with cell numbers in clusters estimated by multiplying by 2.5, 4, 10.5, and 32 times the number of clusters of 2-3, 4, 5-16, and 16+ cells, respectively. The volume of the ODRP-3 controls was then subtracted from all groups to assess deviation from the control culture. Culture supernatants from all treatments were analyzed for protease activity by zymography.

*Assessment of the effects of homogenate supplementation from different Crassostrea species and C. virginica populations on P. marinus infectivity*

Oyster tissue homogenates were produced from pooled tissues of three oysters from each of the test groups, *C. virginica* LA, *C. virginica* TG, *C. virginica* XB, *C. virginica* WA, *C. gigas*, and *C. ariakensis*. Cultures of the P-1 isolate of *P. marinus* were seeded at  $10^6$  cells per mL in  $75 \text{ cm}^2$  flasks containing 50 mL of ODRP-3 medium without supplementation, or supplemented at 0.25 mg/mL with homogenates from each of the oyster groups. This supplementation level was chosen based on prior observations of alterations in *P. marinus* culture cell count, morphologic characteristics and protease production. Cultures were maintained in a humidified incubator at  $27^\circ\text{C}$  under 5%  $\text{CO}_2$  for four weeks. Cells were then harvested for oyster challenge, and the culture supernatants retained for zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size was estimated by measurement of ten cells per



treatment using an ocular micrometer. Thirty *C. virginica* WA oysters per group were subjected to experimental infection.

## Results

### *Perkinsus marinus* clonal isolates display a range of infectivity and protease expression

*Perkinsus marinus* clonal isolate cell diameters were consistent within each group, ranging from 2  $\mu\text{m}$  (SC3-2) to 4  $\mu\text{m}$  (LA10-1, MA2-11, P-1). The cell size of the HVA-18 isolate was more variable, ranging from 2 to 6  $\mu\text{m}$ . All cells in the cultures were single trophozoites, with little to no clustering of cells. There were significant differences in the oyster *P. marinus* body burdens four weeks after experimental infection (Fig. 1a), with all groups significantly greater than SC3-2. The LA10-1, P-1, and HVA-18 isolates were not significantly different. The MA2-11 isolate was significantly more infective than both the P-1 and the HVA-18 isolates. There were no mortalities, and all sham injected oysters were negative for *P. marinus* infection.

Low molecular weight proteases were most strongly expressed in the culture supernatants of HVA-18 and MA2-11 (Fig. 1b). The LA10-1 and P-1 isolates also showed some weak low molecular weight protease activity. High molecular weight proteases were most prominent in the HVA-18 and P-1 culture supernatants. The SC3-2 culture supernatant did not have any apparent protease activity. The FBS proteins, which can be visualized as dark bands on the zymogram, were degraded to some extent in the MA2-11 and P-1 cultures, and were completely degraded in the HVA-18 culture.

### *Infectivity of P. marinus* isolate P-1 is increased by media supplementation with oyster plasma or tissue homogenate

Unsupplemented cells and cells supplemented with plasma were similarly sized, with most cells being 5 to 6  $\mu\text{m}$ . The homogenate supplemented cells were larger, at ~10  $\mu\text{m}$ , and had frequent multicellular tomont structures, as well as numerous clusters of

adherent cells. These were not seen in the plasma-supplemented or the unsupplemented cultures. Gelatin zymograms demonstrated production of low molecular weight proteases in supplemented cultures, with greater activity induced by homogenate supplementation (Fig. 2b). The media controls did not demonstrate any proteolytic activity.

Infection levels at four weeks were significantly higher in oysters challenged with *P. marinus* from supplemented cultures as compared with those grown in ODRP-3 medium (Fig. 2a). Sham injected controls were all negative for *P. marinus* infection. There were seven, nine, ten, and three oyster mortalities in the homogenate, plasma, unsupplemented and sham injected groups through the course of the experiment. The cause of the mortalities could not be determined due to rapid degradation of the oyster tissues.

*Oyster tissue homogenate alters P. marinus proliferation, size, morphology, and protease expression*

Oyster homogenate supplements from all three oyster species resulted in the occurrence of multicellular clusters, which were resistant to disaggregation by pipetting (Figs. 3, 4). While no shared exterior wall could be visualized, these multicellular clusters were often compressed, and were similar in appearance to tomonts. P-1 isolate total cell counts were diminished by oyster homogenate supplementation, regardless of the oyster species (Fig. 3). This effect was dose-dependent, with some effect seen even at the lowest supplementation levels. Cells supplemented with *C. ariakensis* homogenate at 1.0 mg/mL appeared to be non-viable, as assessed by neutral red uptake. Supplementation with fetuin was associated with minor diminishment in the number of cells at low and intermediate concentrations, and a moderate increase in cell count at the 1.0 mg/mL supplementation level (Fig. 3). Cultures supplemented with FBS behaved similarly to fetuin; however, there was a large increase in proliferation at the highest

supplementation levels. When supplemented at 1.0 mg/mL with FBS, the *P. marinus* cell count was approximately 2.5-fold higher than in the ODRP-3 control.

Most multicellular clusters observed in the homogenate supplemented medium consisted of 5-16 aggregated cells. The degree of cell clustering was dose-dependent, and was typically maximal at intermediate supplement concentrations. The *C. virginica* WA supplemented cultures had a peak in the number of clusters at 0.11 mg/mL and maintained clusters to the highest dose tested. *Crassostrea ariakensis* and *C. gigas* supplemented cultures had a peak in cell clustering at the lower supplementation level of 0.037 mg/mL. There was a precipitous decline in the occurrence of these clusters at the 0.33 mg/mL dose for *C. ariakensis* and at the 1.0 mg/mL dose for *C. gigas*. Neither fetuin nor FBS supplementation was associated with formation of multicellular clusters.

Homogenate supplementation from all three oyster species also resulted in a dose-dependent increase in the mean *P. marinus* cell size (Figs. 4, 5). Cells supplemented at 1.0 mg/mL had an over threefold larger diameter than unsupplemented cells. Neither FBS nor fetuin supplementation resulted in a change in the cell size as compared with the ODRP-3 control. The calculated cell "pellet volume" was greater than unsupplemented cultures at the highest supplementation levels for FBS, fetuin, and *C. virginica* WA supplemented cultures (Fig. 5b). Both *C. ariakensis* and *C. gigas* supplemented cultures had volumes greater than unsupplemented cultures only at the 0.33 mg/mL supplementation level.

A dose-dependent increase in low molecular weight protease production was observed, as expected, in cultures supplemented with *C. virginica* WA oyster homogenate (Fig. 6). Homogenates from the *P. marinus*-tolerant *C. gigas* oysters did not induce low molecular weight proteases. The *C. ariakensis* oyster homogenate induced low molecular weight proteases only at the 1.0 mg/mL supplementation level, and these bands were faint and difficult to visualize on the zymogram. Both FBS and fetuin induced low molecular weight proteases at supplementation levels of 0.33 mg/mL and

above. Oyster supplement protease activity was seen at approximately 60 kDa in the homogenate supplemented cultures and media controls, especially at the highest dosage levels. This protease activity was fully inhibited by the addition of 10 mM EDTA to the incubation buffer, and is therefore assumed to represent oyster metalloprotease activity similar to that described by Ziegler et al. (Ziegler et al., 2002).

*Oyster tissue homogenates from different oyster species and populations cause increased infectivity*

*Perkinsus marinus* isolate P-1 cell cultures were supplemented with 0.25 mg/mL oyster homogenate from *C. virginica* LA, *C. virginica* WA, *C. virginica* TG, *C. virginica* XB, *C. ariakensis*, and *C. gigas* oysters. Cells from *C. ariakensis*, *C. virginica* LA, and *C. virginica* TG supplemented cultures had similar mean size of 7.6 to 7.7  $\mu\text{m}$ . Cells supplemented with *C. virginica* XB were slightly larger, with a mean size of 9.0  $\mu\text{m}$ , and those supplemented with *C. virginica* WA were smaller, at 5.2  $\mu\text{m}$ . Unsupplemented cultures had a mean cell size of 4.1  $\mu\text{m}$ . All homogenate supplemented cultures formed large clusters of cells resistant to disaggregation by pipetting. No clusters were noted in the unsupplemented culture.

All homogenate supplements tested in the infection study resulted in increased body burdens; however, only those oysters infected with cells pulsed with *C. ariakensis* or *C. virginica* LA homogenate had body burdens significantly different than that of the ODRP-3 control (Fig. 7a). There was one mortality in the *C. virginica* LA group, two in the *C. virginica* XB group, and three each in the *C. ariakensis* and *C. virginica* WA groups. Sham injected oysters were negative for *P. marinus* infection, and experienced no mortalities. Cells grown in the *C. gigas* homogenate supplemented cultures were not viable at the time of infection, as assessed by neutral red uptake. The cause of this is not known, as concentrations of *C. gigas* homogenate both above and below this level were used in other experiments without loss of culture viability. Gelatin zymograms

Figure 1. Infectivity level and secreted protease profiles of selected *P. marinus* isolates.

(a) Mean body burden  $\pm$  standard error of oysters infected with *P. marinus* isolates. Cells were grown for three weeks in Dungan's medium at 27°C in 5% CO<sub>2</sub>, then injected at 10<sup>6</sup> cells per oyster per day on days 1, 3, and 5 into twenty *C. virginica* ME oysters per group. After four weeks, the oysters were sacrificed and total *P. marinus* body burden assessed, and log<sub>e</sub> transformed for statistical analysis. Groups with different numbers were significantly different ( $p < 0.001$ ).

(b) Gelatin zymogram of 20  $\mu$ L of culture supernatant from *P. marinus* isolate cultures used in the above infection trial. Non-reducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE were incubated 3 hours at 37°C in 0.1 M Tris HCl, pH 8.0, then stained with Coomassie brilliant blue G-250. Molecular weights are in kilodaltons.

Figure 1.

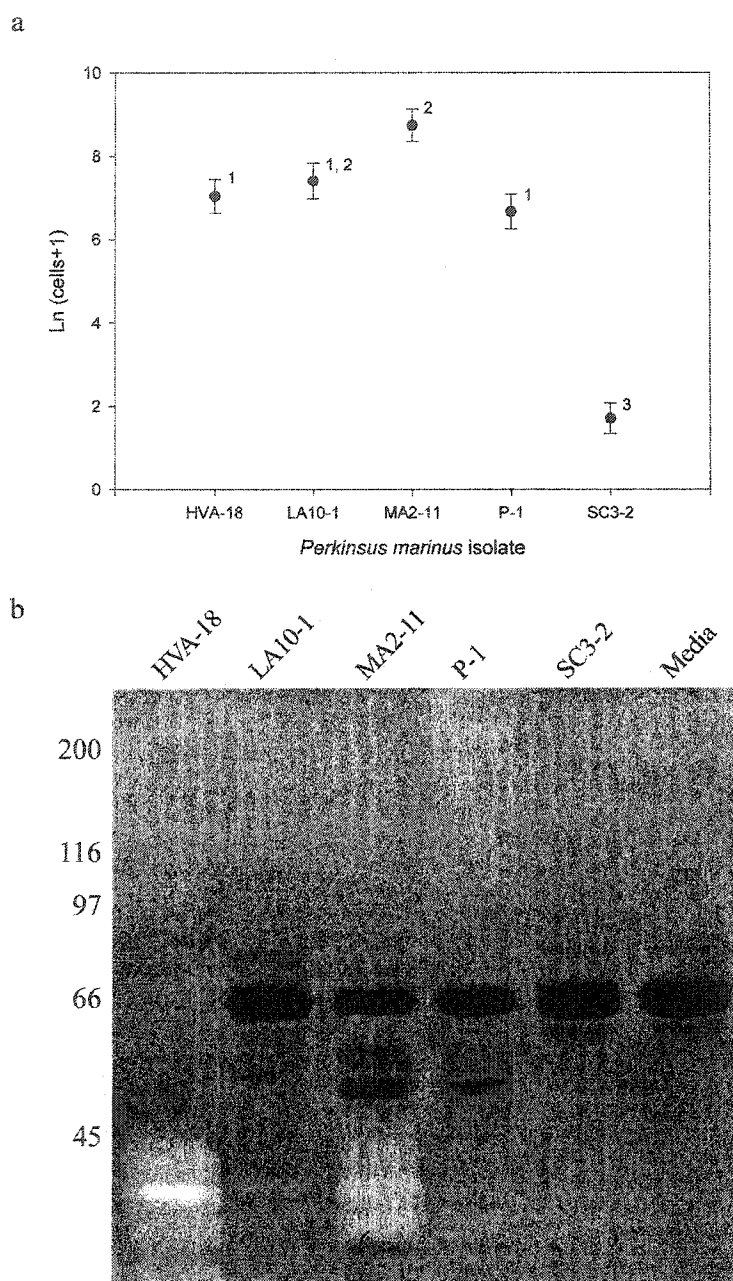


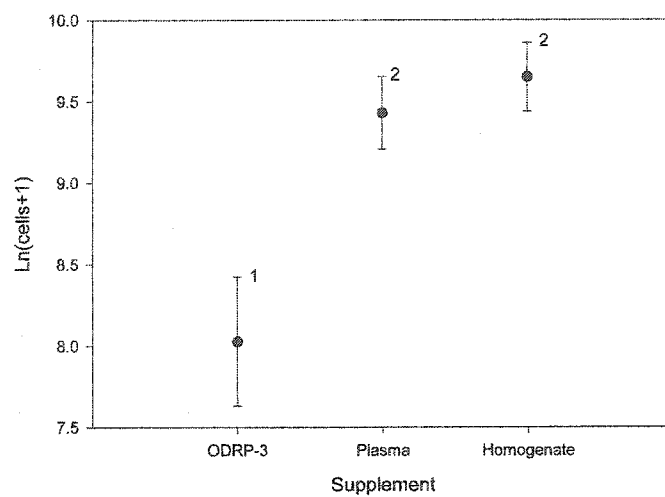
Figure 2. Infectivity level and secreted protease profiles of *P. marinus* isolate P-1 in plasma and homogenate supplemented cultures.

(a) Mean body burden  $\pm$  standard error of oysters infected with *P. marinus* isolate P-1 grown for four weeks in ODRP-3 medium without supplementation or supplemented with 0.3 mg/mL of oyster plasma or homogenate at 27°C in 5% CO<sub>2</sub>, then injected at 10<sup>6</sup> cells per oyster per day on days 1, 3, and 5 into thirty *C. virginica* ME oysters per group. After four weeks, the oysters were sacrificed, the total *P. marinus* body burden assessed and log<sub>e</sub> transformed for statistical analysis. Groups with different numbers were significantly different (p<0.001).

(b) Gelatin zymogram of 20 µL of culture supernatant from supplemented and unsupplemented *P. marinus* cultures from the above infection trial. Non-reducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE were incubated 3 hours at 37°C in 0.1 M Tris HCl, pH 8.0, then stained with Coomassie brilliant blue G-250. Molecular weights are in kilodaltons.

Figure 2.

a



b

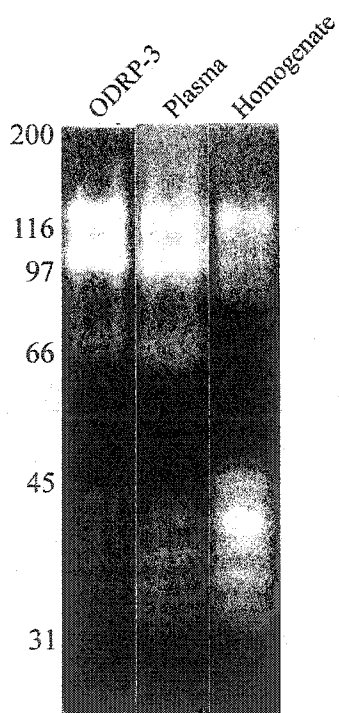




Figure 3. Mean counts of single trophozoites, multicell clusters, and calculated total cell number (see text) from *P. marinus* isolate P-1 cultures supplemented with a range of doses of tissue homogenate from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), *C. gigas* (Cg) oysters, or with fetal bovine serum (FBS) or fetuin. Cultures were incubated for six weeks at 27°C in 5% CO<sub>2</sub>, resuspended by repeated pipetting and enumerated by hemacytometer. Counts are the mean of duplicate cultures from each of five separate oysters tested. FBS and fetuin were tested in duplicate. Standard error bars, ANOVA significance and multiple comparison results are shown for single trophozoites. Groups with different numbers were significantly different.

Note: Homogenates from two of five *C. gigas* oysters were not in sufficient concentration to allow 1.0 mg/mL supplementation; therefore, this dose was omitted for these oysters.

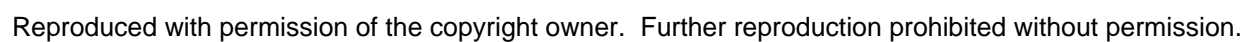


Figure 4. Light micrograph (neutral red stain, 1000X) of *P. marinus* isolate P-1 cultures supplemented with a range of tissue homogenate concentrations from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), *C. gigas* (Cg) oysters or with fetal bovine serum (FBS) or fetuin. Cultures were incubated for six weeks at 27°C in 5% CO<sub>2</sub>, resuspended by repeated pipetting, and digitally photographed.

Figure 4.

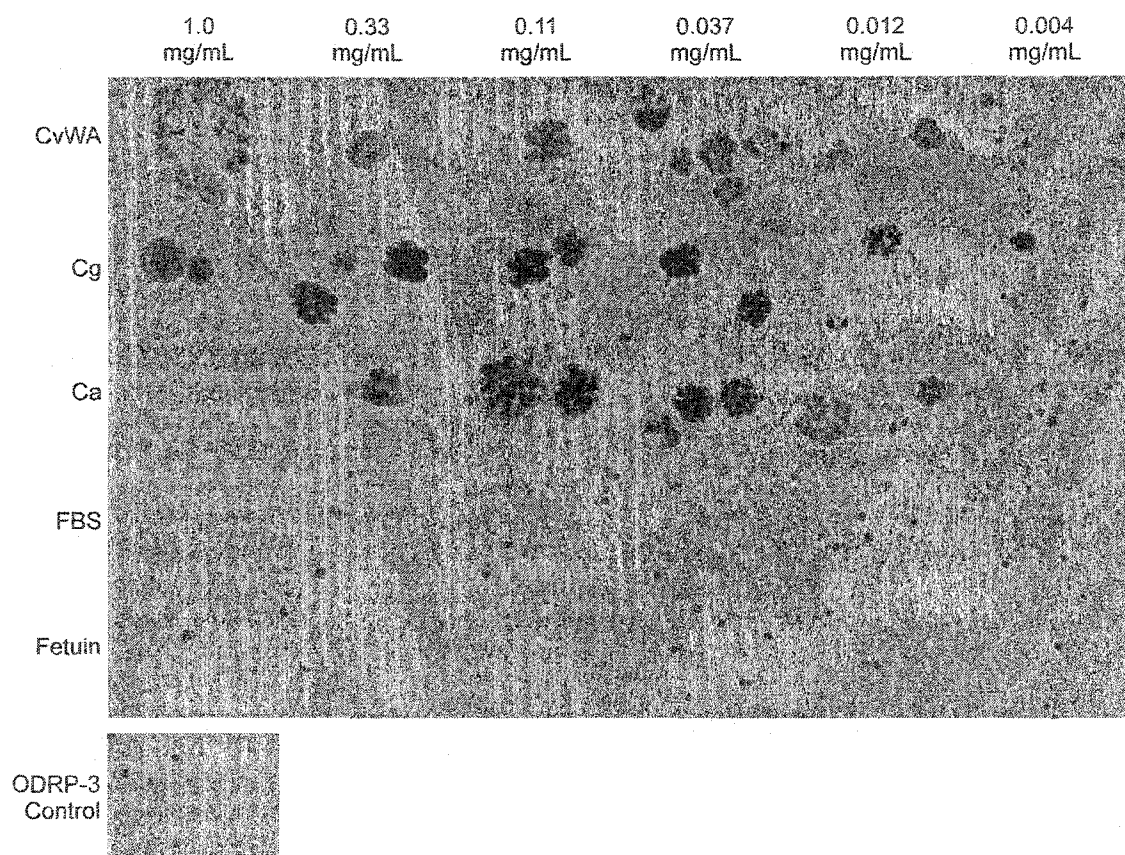


Figure 5. Supplemented culture cell size and deviation of pellet volume from unsupplemented control cultures.

(a) Cell size  $\pm$  standard deviation from *P. marinus* isolate P-1 cultures supplemented with a range of tissue homogenate concentrations from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), *C. gigas* (Cg) oysters or with fetal bovine serum (FBS) or fetuin.

Cultures were incubated for six weeks at 27°C in 5% CO<sub>2</sub>, resuspended by repeated pipetting and measured at 400X using an ocular micrometer. Sizes of ten trophozoites were measured in duplicated culture wells supplemented with homogenates of five separate oysters per group. *C. gigas* (Cg) measurements included only three oyster supplements at the 1.0 mg/mL dose.

(b) Deviation of calculated pellet volume (see text) of *P. marinus* isolate P-1 grown in supplemented cultures from the mean unsupplemented pellet volume.

Figure 5.

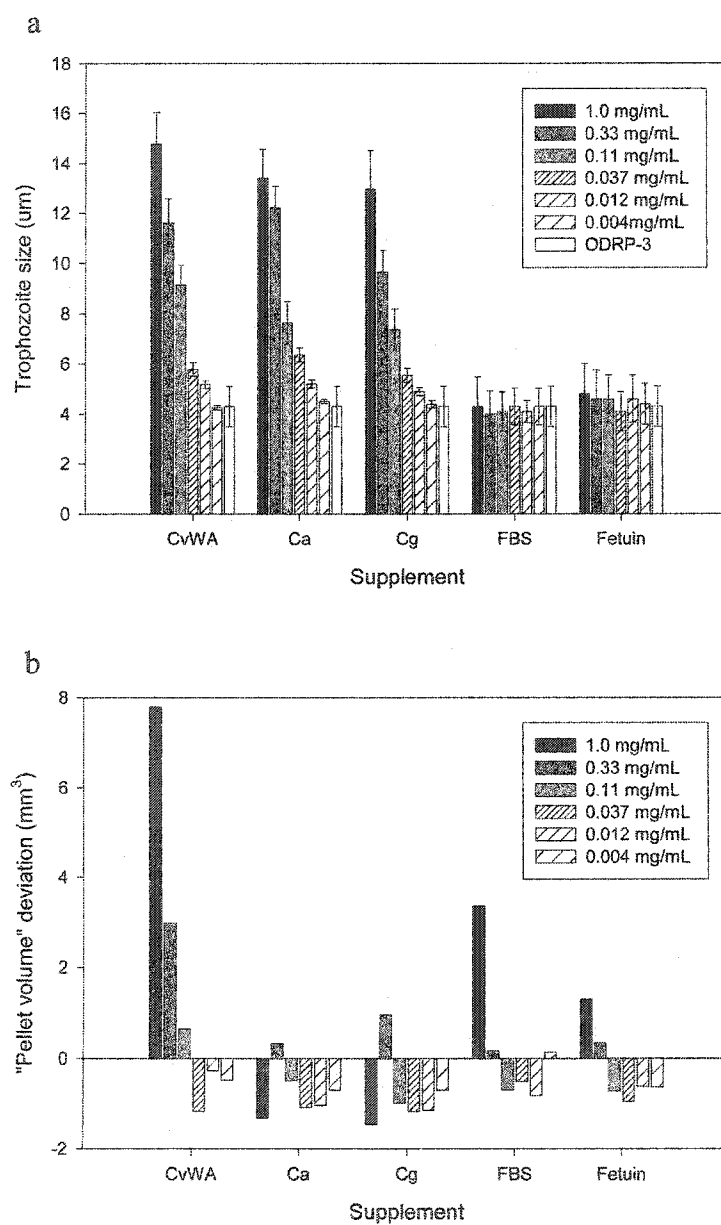


Figure 6. Gelatin zymogram of 20  $\mu$ L of culture supernatant from *P. marinus* isolate P-1 cultures supplemented with 1.0 mg/mL (a), 0.33 mg/mL (b), 0.11 mg/mL (c), 0.037 mg/mL (d), 0.012 mg/mL (e), or 0.004 mg/mL (f) of tissue homogenate from *C. virginica* WA (CvWA), *C. ariakensis* (Ca), *C. gigas* (Cg) oysters or with fetal bovine serum (FBS) or fetuin. ODRP-3 is the unsupplemented control. Cultures were grown for six weeks at 27°C in 5% CO<sub>2</sub>. Non-reducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE were incubated 3 hours at 37°C in 0.1 M Tris HCl, pH 8.0, then stained with Coomassie brilliant blue G-250. Molecular weights are in kilodaltons.

Figure 6.

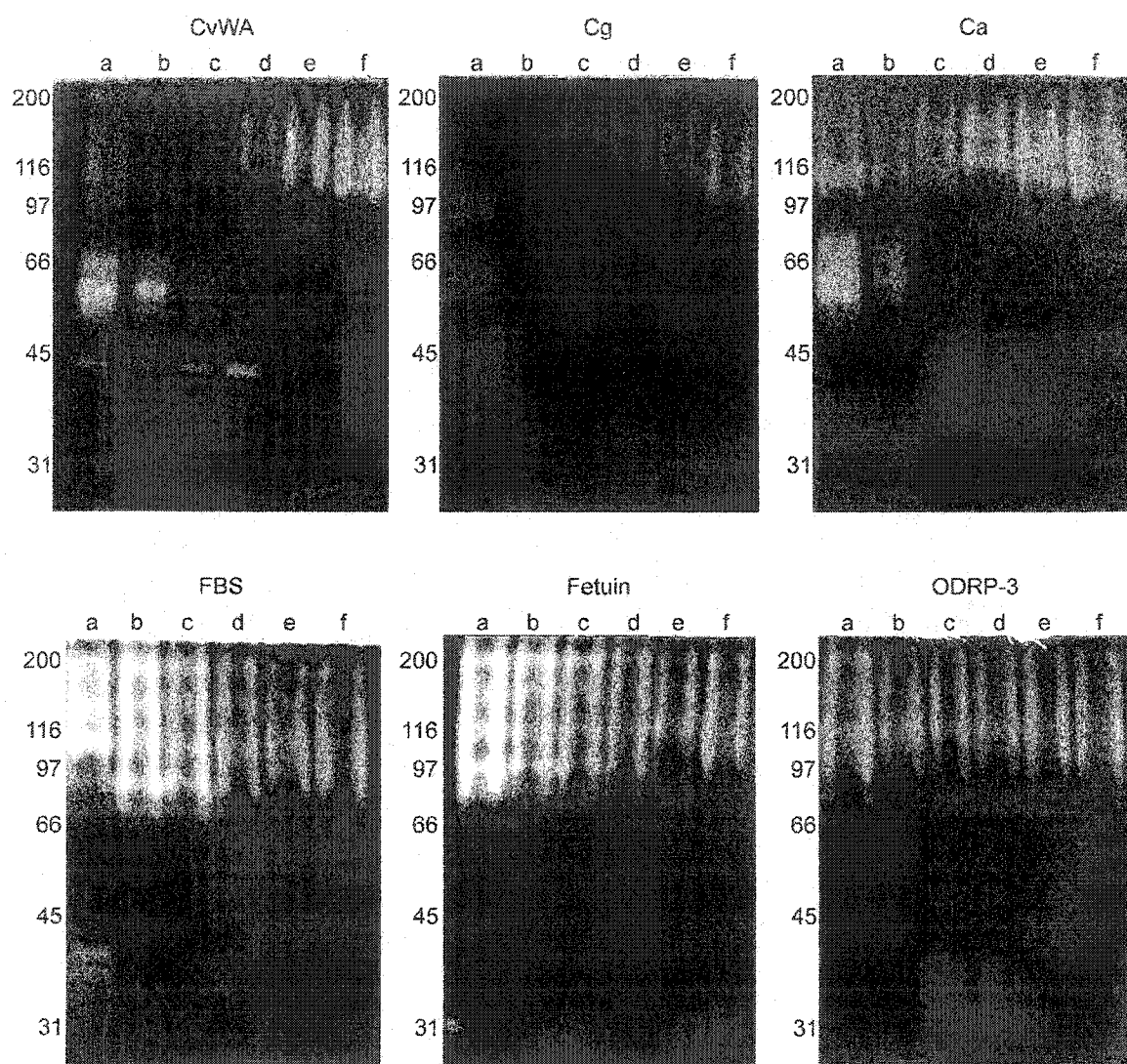


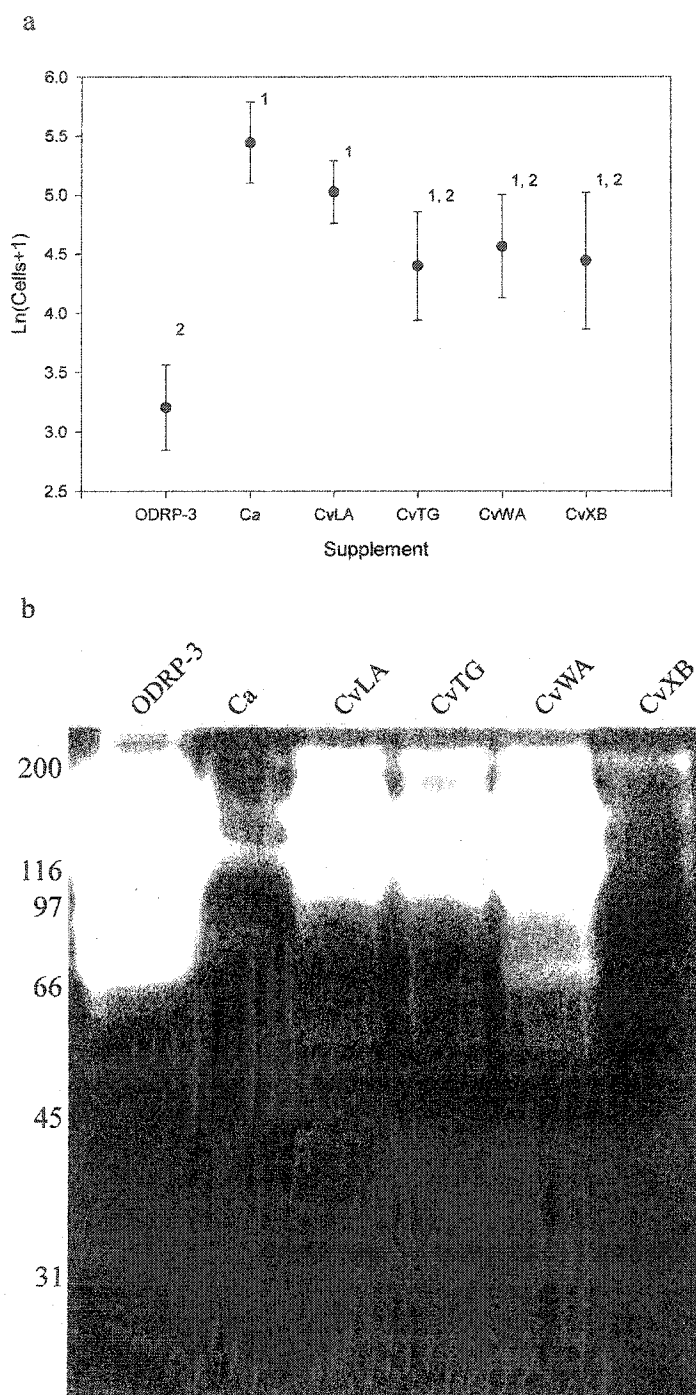


Figure 7. Infectivity level and secreted protease profiles of *P. marinus* isolate P-1 grown in homogenate supplemented cultures.

(a) Mean body burden  $\pm$  standard error of oysters infected with *P. marinus* isolate P-1 grown for four weeks at 27°C in 5% CO<sub>2</sub> in ODRP-3 medium without supplementation or supplemented with 0.25 mg/mL of homogenate from *C. ariakensis* (Ca), or from *C. virginica* oysters from Louisiana (*C. virginica* LA), Tangier Sound, Chesapeake Bay, VA (CvTG), Washington state (CvWA), or from the CROSBreed program (CvXB). 10<sup>6</sup> cells per oyster per day were injected on days 1, 3, and 5 into thirty *C. virginica* WA (CvWA) oysters per group. After four weeks, the oysters were sacrificed and total *P. marinus* body burden assessed and log<sub>e</sub> transformed for statistical analysis. Groups with different numbers were considered significantly different (p=0.007).

(b) Gelatin zymogram of 20 µL of culture supernatant from *P. marinus* isolate P-1 grown for four weeks at 27°C in 5% CO<sub>2</sub> in ODRP-3 medium without supplementation or supplemented with 0.25 mg/mL of homogenate as described above. Non-reducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE, incubated overnight at 37°C in 0.1 M Tris HCl, pH 8.0 with 10 mM EDTA included to limit oyster metalloprotease activity on the zymogram that obscured some of the lower intensity *P. marinus* serine proteases. The gels were stained with Coomassie brilliant blue G-250. Molecular weights are in kilodaltons.

Figure 7.



demonstrated induction of low molecular weight protease in the *C. virginica* LA culture only (Fig. 7b). The unsupplemented ODRP-3 culture displayed the typical high molecular weight proteases. High molecular weight protease activity in the *C. ariakensis* and the *C. virginica* XB cultures was greatly diminished. No protease activity was detected in the media controls.

## Discussion

Neither the physical nor the biochemical mechanisms of *P. marinus* infectivity or virulence are understood; however, this parasite can be rapidly attenuated by *in vitro* culture (Ford et al., 2002). The mechanism of attenuation is not known, but may be due to the lack of critical growth, differentiation, or virulence-induction factors provided by the host. Previous attempts to restore virulence by passage of cultured parasites through a host oyster did not succeed, possibly due to the brief reculture required to produce an inoculum for challenge (Ford et al., 2002), suggesting attenuation occurs quite rapidly.

### *Perkinsus marinus* isolates

Non-clonal *P. marinus* isolates have been shown to vary in virulence, with Atlantic coast isolates being more virulent than those from the Gulf of Mexico (Bushek and Allen, 1996a; Bushek and Allen, 1996b). In this study, isolates of *P. marinus* that were chosen based on their assignment by genetic markers into distinct genotypes (Reece et al., 1997) displayed differences in infectivity as evidenced by the four week body burden (Fig. 1a). The South Carolina isolate, SC3-2, was associated with the lowest level of infection. Eight of the experimental oysters infected with SC3-2 were negative for *P. marinus* infection at week four, and the highest body burden in any oyster infected with SC3-2 was 96 cells.

It is possible that the small size of the SC3-2 isolate ( $\sim 2\mu\text{m}$ ) rendered the cells less likely to be phagocytosed by mantle cavity hemocytes or ingested during feeding.

The efficiency of particle filtration by oysters varies directly with the size of the particle (Haven and Morales-Alamo, 1970; Palmer and Williams, 1980; Riisgard, 1988), and previous studies have demonstrated that ingestion of *P. marinus* is one probable route of parasitic infection (Alvarez et al., 1992). While it has also been suggested that hemocytes in the mantle cavity may be responsible for transport of *P. marinus* into the oyster (Perkins, 1976b; Chintala et al., 2002), it is not known whether there are size-mediated differences in rates of phagocytosis and transport of *P. marinus* into the oyster by hemocytes. Given these results, there is an indication that *P. marinus* cell size could be a factor in its infectivity.

#### *Supplementation and infectivity*

The possibility that cell size is a determinant of infectivity was not supported, however, by the results of the other infection trials. When *P. marinus* P-1 isolate cells were grown in the presence of oyster plasma supplement, the cell size and morphology were similar to unsupplemented cultures. The body burden after experimental infection, however, was significantly higher, and was statistically similar to that seen with cells enlarged by culture in homogenate supplemented media. Additionally, despite inducing similar body burdens (Fig. 7a), P-1 cells from cultures supplemented with *C. virginica* WA or *C. virginica* XB homogenates had markedly different mean sizes (5.2 $\mu$ m and 9.0 $\mu$ m, respectively). The cells with the greatest infectivity were those supplemented with *C. ariakensis* homogenate, and they were very similar in size (mean ~7.6  $\mu$ m) to two other groups of lesser infectivity, *C. virginica* LA and *C. virginica* TG. In this latter infection study, infections were initiated in *C. virginica* WA oysters, and it should be noted that the magnitude of the body burdens at four weeks was markedly lower than was seen in the earlier trials using *C. virginica* ME oysters. The cause of this is not known, but it is assumed that the *C. virginica* WA oysters may be more resistant to *P. marinus*.

infection. This is particularly interesting because this population of oysters has been isolated from *P. marinus* for numerous generations (Muñoz et al., 2003).

The ability to increase parasite infectivity appears to be a property specific to host plasma and tissue homogenates. Supplementation of cell cultures with FBS in Dungan's medium did not cause the increases in infectivity seen with oyster homogenate or plasma supplementation. The mean four week body burden from *C. virginica* ME oysters infected with P-1 grown in Dungan's medium with FBS was 2,327 cells (Fig. 1a), while those from *C. virginica* ME oysters infected with P-1 grown in plasma and homogenate had mean burdens of 18,975 and 25,311, respectively (Fig. 2a).

#### *Morphology, size, and proliferation*

Oyster homogenate supplements were the most effective inducers of morphological changes, altered protease profiles, and increased infectivity. Supplementation of media with homogenates from either susceptible or tolerant oyster species resulted in a marked reduction in cell proliferation at increasing doses (Fig. 3). At the 1.0 mg/mL supplementation level for both *C. ariakensis* and *C. gigas* homogenates, there was a decrease in cell number below the seeding concentration, with the *C. ariakensis* cells appearing to be non-viable (Fig. 4). At high supplementation levels, the diminution was similar between all oyster species supplements. At the lowest two supplement doses, the suppressive effect was more pronounced in the disease tolerant *C. ariakensis* and *C. gigas* oysters (Fig. 3). The suppression of proliferation was only associated with oyster product supplementation. Fetuin supplementation caused a minor decrease in cell proliferation that was consistent at lower supplement doses. Both the 0.33 mg/mL and 1.0 mg/mL supplementation levels were similar to the ODRP-3 control. FBS behaved similarly, except that there was a large increase in proliferation at the 0.33 mg/mL, and especially at the 1.0 mg/mL, supplementation levels. This is in

general agreement with the findings of Gauthier and Vasta (1995), who previously described the proliferative effects of FBS and fetuin on *P. marinus* cultures.

Since increasing supplementation levels with oyster homogenates are associated with decreasing cell counts (Fig. 3) and increasing cell size (Figs. 4, 5a), it was desirable to understand their combined effects using the calculated “pellet volumes.” The results were not surprising for the FBS and fetuin cultures. Cell sizes did not change over the range of supplementation, and there are no cell clusters, thus the pellet volumes paralleled the cell counts. Supplementation with any oyster homogenate was associated with a reduced pellet volume at the three lowest dosage levels (Fig. 5b). At 0.11 mg/mL, the *C. virginica* WA supplemented cells had a pellet volume in excess of the ODRP-3 control. At 0.33 mg/mL, this effect was even more pronounced in the *C. virginica* WA oyster group, and was seen in both the *C. gigas* and *C. ariakensis* supplemented cultures, despite the continuing decrease in cell count. At 1.0 mg/mL, the *C. virginica* WA culture pellet volume was nearly 8 mm<sup>3</sup> larger than the ODRP-3 control. At this highest supplementation level, the *C. gigas* pellet volume again fell below ODRP-3 control, as did that of the non-viable *C. ariakensis* culture.

*In vivo*, *P. marinus* cells range in size from 3.9 to 11.6 µm, with a mean of 5.5 µm (Perkins, 1996), similar to that elicited by media supplementation with oyster products. The association of larger cell size with lower replication rate may indicate there is a regulation of mitosis by exposure to host products. *Perkinsus marinus* appears to regulate its replication, especially at high infection intensities, such that the parasite burden remains at non-lethal levels (Saunders et al., 1993). This regulation may be based on energy limitations (Choi et al., 1989). The decreased *in vivo* replication rate could also be mediated by an ability of *P. marinus* to sense cell density and decrease replication rate accordingly, as has been documented in certain quorum-sensing bacteria (Withers and Nordstrom, 1998). This does not seem likely, as previous studies have found that the presence of *P. marinus* cells and extracellular products has a positive effect on *in vitro*

replication rate (Gauthier and Vasta, 1995; Bushek et al., 2000). Alternatively, this change may represent a partial transition to the prezoosporangium life stage. In fluid thioglycollate medium, trophozoite maturation to prezoosporangia (hypnospores) is associated with thickening of the cell wall and marked enlargement of the vacuole, resulting in cell enlargement to 30-80  $\mu\text{m}$  (Perkins, 1996). While this enlargement is considerably greater than is seen in supplemented cultures, it is nonetheless possible that supplementation results in the preliminary phases of transition to this life stage.

The current data suggest that there may be a molecular signal present in all three oyster species tested which regulates *P. marinus* proliferation. Since this effect occurs only with tissue homogenates, the signaling molecule could be a product of tissue degradation. In that case, an increase in the number of *P. marinus* cells during infection and the associated tissue damage from proteolytic activity could signal *P. marinus* to decrease the replication rate and allow the oyster to survive longer in the parasitized state. This possibility is supported by the greater potency of the homogenate effect from the tolerant *C. gigas* and *C. ariakensis* oysters on *P. marinus* cell replication (Fig. 3).

#### *Protease induction*

The initial work investigating the induction of proteases by oyster homogenate (MacIntyre et al., 2003) was largely confirmed by the dose-response experiment, though there was some induction of low molecular weight proteases by *C. ariakensis* homogenate, FBS, and fetuin at doses above those assessed in the earlier research. The induction of low molecular weight proteases does correlate with infectivity in cultures supplemented with oyster plasma and homogenate (Figs. 3, 4). It is tempting to correlate increased infectivity with induction of these proteases under exposure to conditions that more closely mimic the *in vivo* environment. The strongest induction of low molecular weight proteases and the highest body burdens were seen in the cells supplemented with homogenate. Similarly, the intermediate level of protease production by plasma

supplemented cells was associated with a slightly, though not significantly, lower body burden. Both were more virulent than the ODRP-3 control, which produced little to no low molecular weight protease.

The relationship between induction of low molecular weight proteases and increased infectivity was not consistent in the other experiments. In the clonal isolates grown in Dungan's medium, the SC3-2 isolate was the least virulent, and demonstrated little to no protease activity in the culture supernatant (Figs. 1, 2). The LA10-1 and P-1 isolate also had lower levels of protease expression in general, and weak low molecular weight protease expression in particular, and were more virulent than SC3-2. The HVA-18 isolate had infectivity similar to LA10-1 and P-1, but had the greatest amount of low molecular weight protease expression. The most virulent isolate, MA2-11, had a level of low molecular weight protease expression that was intermediate between HVA-18, and LA10-1 and P-1. It is interesting to note that the two Virginia isolates, HVA-18 and P-1 more strongly expressed the series of high molecular weight proteases.

When the P-1 isolate was exposed to 0.25 mg/mL of various oyster homogenates, the *C. virginica* LA and *C. ariakensis* supplemented cultures were the most infective (Fig. 7a). While the *C. virginica* LA supplementation induced low molecular weight proteases, *C. ariakensis* supplementation did not. In addition, the *C. ariakensis* supplemented media elicited decreased level of high molecular weight protease activity (Fig. 7b). Given these varied results, there does not appear to be a consistent correlation between the *in vitro* induction of low molecular weight proteases by homogenate supplementation and infectivity as measured by week four body burden.

The origin of the low molecular weight proteases and their relationship to the higher weight forms is not clear. There often appears to be a diminishment in high molecular weight protease activity associated with increases in the appearance of the low molecular weight forms. It is not known if these changes in the protease profile are due to changes at the transcriptional, translational, post-translational, or secretory level.



Previous research has demonstrated that the low molecular weight forms can not be created from the high molecular weight forms by incubation in the presence of host products (MacIntyre et al., 2003), indicating that the low molecular weight forms are likely not originated by proteolytic cleavage of a multimeric form.

The supplementation of *P. marinus* cultures with oyster products from either disease-tolerant or susceptible oysters modulates cell proliferation, size, and morphology, as well as protease expression and infectivity. Many of these effects cannot be reproduced using common bovine media supplements. None of these varied effects appear to be linked in all cases, indicating that exposure to oyster products likely causes a complex alteration in *P. marinus* physiology resulting in a multitude of phenotypic and functional changes.

### Chapter 3

#### **Homogenates of selected *Crassostrea virginica* organs and tissues differentially affect *Perkinsus marinus* *in vitro* proliferation, morphology, and protein expression**

##### **Abstract**

*Perkinsus marinus* is an alveolate parasite of the eastern oyster, *Crassostrea virginica*. Marked phenotypic changes have been elicited in previous studies by culture medium supplementation with whole oyster homogenate. These changes include alteration in cell proliferation and size, induction of replication by palintomic fission, and alteration of the expression pattern of secreted serine proteases. To determine if specific tissues could differentially elicit some or all of these changes, a protein-free, defined medium was supplemented with homogenates from specific oyster organs or tissues. Generally, medium supplementation reduced proliferation; however, supplementation with low concentrations of adductor muscle tissue induced proliferation levels greater than is seen in the unsupplemented cultures. Supplementation with whole oyster homogenate and, more specifically, the homogenate of the digestive gland and gonad, reduced the total cell count, but favored reproduction by palintomic, rather than binary, fission. Protease activity in the parasite extracellular products was generally decreased in supplemented cultures, except in gill/mantle supplemented cultures, where there was apparent induction of proteolytic activity. Zymograms indicated that low molecular weight proteases were upregulated by heart- and adductor muscle-derived supplements.

## Introduction

The protozoan parasite, *Perkinsus marinus*, causes a chronic wasting disease which is partially responsible for the decline of oyster (*Crassostrea virginica*) populations along the eastern seaboard of the United States (Burreson and Ragone-Calvo, 1996; Ford, 1996). The exact mechanisms of parasite differentiation and virulence within the host are not known; however, *P. marinus* is known to exist *in vivo* primarily as a trophozoite, progressing from a small, immature to a mature “signet ring” form, so named for the microscopic appearance of its large vacuole and offset nucleus. Mature trophozoites can form a tomont, a reproductive structure that undergoes palintomic fission to form 4 to 64 immature trophozoites within the parental cell wall (Perkins, 1996). *Perkinsus marinus* can also form motile zoospores, again by palintomic fission, which exit through a discharge tube and pore structure formed on the wall of the enlarged parental trophozoite, the zoosporangium (Perkins, 1996).

*In vitro*, cellular proliferation of *P. marinus* in protein-free medium (ODRP-3; LaPeyre and Faisal, 1997) occurs solely by binary fission, and zoosporulation is not seen. Supplementation of that medium with oyster products is associated with changes in cell proliferation, and with the appearance of cellular morphologies and the tomont reproductive stage typically found *in vivo* (Gauthier and Vasta, 2002; MacIntyre et al., 2003; Earnhart et al., 2004). High supplement concentrations have also been associated with a partial transition to a prezoosporangium stage (Earnhart et al., 2004; See this dissertation, Chapter 6).

Medium supplementation also causes changes in the expression pattern of *P. marinus* extracellular products (ECP), which may contain one or more virulence factors, including serine proteases (Garreis et al., 1996; LaPeyre et al., 1996; Anderson, 1999; Oliver et al., 1999; Oliver et al., 2000). Medium supplementation changes the pattern of ECP serine protease expression, with upregulation of a set of low molecular weight (30-45 kDa) proteases and concurrent downregulation of some higher molecular weight

proteases (MacIntyre et al., 2003; Earnhart et al., 2004). Another ECP constituent, p107, is a potential virulence factor and is expressed at greatly reduced levels in the presence of whole oyster supplements (See this dissertation, Chapter 6).

Medium supplementation also alters the interaction of the parasite with the host during artificial infection. The virulence of *Perkinsus marinus* cultures is rapidly attenuated during *in vitro* culture (Ford et al., 2002); however, this attenuation has been partially reversed by medium supplementation with either oyster plasma or whole oyster homogenate prior to infection (Earnhart et al., 2004). This reversal of attenuation is not consistently associated with alterations in cell size, morphology, or ECP protease expression (Earnhart et al., 2004). The complexity of the responses to oyster product supplementation *in vitro* may be due to an interplay of multiple regulatory signals (e.g. inductive/suppressive) provided by the complex mixture of host products. Efforts to refine or restrict these regulatory signals may lead to a clearer resolution of the molecular cues required for specific physiological or differentiative processes. In this study, we are observing alterations in proliferation and cell morphology as well as changes in ECP composition, induced by incubation with medium supplements derived from specific host organs and tissues.

## Materials and methods

### *Animals*

*Crassostrea virginica* oysters from Washington state (Taylor Resources, Inc., Shelton, WA) were used for all experiments (Muñoz et al., 2003). The oysters were maintained in 1 µm-filtered water (York River, Virginia) and fed commercially produced algae (Shellfish Post-set Formula; Reed Mariculture, San Jose, CA). Water changes were performed twice weekly, and all effluent water was chlorinated prior to release. A subsample of the oysters was confirmed to be negative for *Perkinsus marinus* infection by the total body burden technique (Fisher and Oliver, 1996).

*Dissection of oyster organs/tissues and preparation of medium supplements*

Using a lapidary saw, a small shell notch was made adjacent to the adductor muscle in ten oysters. Approximately 2 mL of hemolymph was removed from the adductor muscle sinuses of each oyster using a syringe fitted with a 25 ga needle. The hemolymph was pooled and centrifuged at  $800 \times g$  for 5 minutes to remove hemocytes. The right valve was then carefully removed to allow dissection of tissues into adductor muscle, heart, gill/mantle, labial palps, and digestive gland/gonad. These tissues, as well as a single whole oyster, were separately homogenized in artificial sea water (20 ppt; Forty Fathoms Marine Mix, Marine Enterprises International, Baltimore, MD) using a glass Tenbroek homogenizer. The homogenates were then centrifuged for 30 minutes at  $2,500 \times g$  at  $4^{\circ}\text{C}$ , and the supernatant withdrawn and centrifuged at  $12,000 \times g$  for an additional 30 minutes at  $4^{\circ}\text{C}$ . These partially clarified homogenates and the cell-free plasma were filtered to  $0.22 \mu\text{m}$  by syringe filters ( $\mu\text{Star LB}$ , Costar, Corning, Inc., Acton, MA), and the protein content assayed by the bicinchoninic acid technique (BCA, Pierce, Rockford, IL).

*Perkinsus marinus* cultures

*Perkinsus marinus* isolate P-1 cells (Faisal et al., 1999) which had been cultured for several years in a chemically-defined, protein-free medium (ODRP-3; LaPeyre and Faisal, 1997; Earnhart and Kaattari, 2003), were grown in the presence of the above described oyster homogenates. ODRP-3 medium was supplemented at 1.5, 0.75, 0.38 and 0.19 mg/mL for each tissue supplement, as well as for plasma and whole oyster homogenate. The heart supplement was not tested at the 1.5 mg/mL concentration due to the small size of the organ. Control wells were not supplemented. *Perkinsus marinus* trophozoites were seeded at  $10^6$  cells/mL in two mL of each supplemented medium in duplicated 24-well tissue-culture plates (Costar 3524, Corning, Inc., Corning, NY). An

additional media control plate was not seeded. The cultures were incubated for six weeks at 27°C in 5% CO<sub>2</sub> in a humidified incubator.

After six weeks, the cells were resuspended by pipetting and counted by hemacytometer, including those contained within tomont structures and in tightly adherent clusters. In addition, an estimate was made of the percentage of particles in the culture which were tomonts or clusters. A thirty-three µL aliquot of each culture was diluted to 200 µL in artificial sea water, applied to a glass slide (Superfrost plus, Fisher Scientific) by cytospin, air dried, methanol fixed, and stained using Hema-3 (Biochemical Sciences, Inc., Swedesboro, NJ) according to the manufacturer's instructions. Cell sizes were measured by ocular micrometer for ten cells per treatment. The remainder of the culture was harvested, centrifuged at 12,000 × g for 10 minutes to remove cells and debris, and used for zymographic and enzyme assays.

#### *Analysis of proteolytic enzymes*

Proteolysis of gelatin substrate was measured both by zymography and by the rate of hydrolysis of fluorescence-quenched gelatin substrate in order to examine protease expression pattern and total gelatinolytic activity, respectively. For zymography, 25 µL of *P. marinus* culture supernatant were electrophoresed under non-reducing conditions by SDS-PAGE (24) using an 8% polyacrylamide separating gel copolymerized with 0.1% porcine gelatin (G-8150, Sigma-Aldrich, Inc.), and a 4% stacking gel. Following electrophoresis, the gels were washed three times for ten minutes in 2.5% Triton-X-100 (BP151, Fisher Scientific, Fairlawn, NJ) to renature the proteins, then once in 0.1 M Tris pH 8.0 (BP152, Fisher Scientific). The gels were then incubated at 37°C in 0.1 M Tris, 10mM EDTA (BP118, Fisher Scientific), pH 8.0 buffer. Two sets of gels were run. One set was incubated for one hour to allow comparison of the relatively active high molecular weight bands, and the other for 16 hours to enable visualization of low molecular weight proteases. The gels were stained with Coomassie brilliant blue G-250

(0.1%; 161-0406, Biorad, Hercules, CA) in 40% methanol, 10% acetic acid, and destained in the same solution, without the stain.

The gelatinolytic activity of proteases from *P. marinus* was also quantified using a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes, Inc., Eugene, OR) (D'Angelo et al., 2001). Seventy  $\mu\text{L}$  of unseeded medium or culture supernatant from each treatment were combined with 10  $\mu\text{L}$  of buffer (0.5M Tris, 2mM  $\text{NaN}_3$ , 100mM EDTA) and 20  $\mu\text{L}$  of DQ gelatin substrate (1.0 mg/mL). Each replicate was analyzed in duplicate wells. The rate of production of fluorescent product was measured using fluorescein filters on a fluorescence plate reader (GENios, Tecan, Durham, NC) while the reaction rate was constant.

#### *Quantification of p107*

The *P. marinus* ECP protein p107 appears to be a target of supplement-induced regulation (Earnhart et al., 2004; See this dissertation, Chapter 6). In order to track changes in the expression of this ECP, it was quantified in each culture supernatant by sandwich ELISA as previously described (See this dissertation, Chapter 6). High-protein-binding 96-well ELISA plates (Costar 3590, Corning, Inc., Corning, NY) were coated with 200 ng of purified monoclonal antibody 5G9 per well in 50  $\mu\text{L}$  of carbonate buffer (15mM  $\text{Na}_2\text{CO}_3$ , 17.4mM  $\text{NaHCO}_3$ , pH 9.7), and residual unbound sites were blocked with 240  $\mu\text{L}$ /well of 1% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TTBS; 50mM Tris, 150mM NaCl, 1mM EDTA, 0.1% Tween-20, pH 7.5). Each culture supernatant was titrated by serial twofold dilutions in 1% BSA in TTBS in duplicate wells and incubated for 1 hour at room temperature. Included on each plate were duplicated serial dilutions of a standard *P. marinus* culture supernatant which was previously determined to contain 200 ng/mL of p107, and a negative control of unseeded culture medium. Following incubation, the plates were washed three times, then incubated with 200 ng of purified, biotinylated monoclonal antibody 1B3 for one

hour at room temperature, followed by a one hour incubation with 100 $\mu$ L of a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin (S5512, Sigma, Inc.). The rate of peroxidase-catalyzed conversion of 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) substrate was measured spectrophotometrically, and p107 was quantified by comparison of sigmoidal curves fitted to the rate data from each dilution series using a four-parameter logistic equation (SigmaPlot, SPSS, Inc., Chicago, IL). The amount of p107 contained in each of the samples was calculated by interpolation at the rate corresponding to 30% of the reaction rate plateau of the standard for that plate (Arkoosh and Kaattari, 1990).

## Results

### *Oyster tissue supplements differentially affect cell count and morphology*

With the exception of the adductor muscle supplement, oyster tissue product supplementation was associated with cell counts below that found in unsupplemented medium (Fig. 1a). The reduced proliferation was most significant above the 0.19 mg/mL supplement level, and was relatively stable with increasing supplementation in all treatments except whole oyster homogenate and plasma. There was marked reduction of proliferation in cultures supplemented with whole oyster homogenate at 1.5 mg/mL. Plasma supplementation was not associated with the same reduction of proliferation with increasing dose. Adductor muscle cultures supplemented at 0.38 and 0.19 mg/mL had cell counts above those in the unsupplemented control.

There were morphological changes induced by supplementation, including increased cell size and the appearance of multicellular tomonths and adherent cell clusters likely representing tomonths that had recently ruptured the outer cell wall (Figs. 1b, 1c, 2). The size changes tended to parallel increases in supplementation level and occurred, to some extent, with all supplements (Fig. 1b). The magnitude of cellular enlargement with increasing supplementation was similar among all tissue supplements, except in those



cultures supplemented with high levels of whole oyster homogenate (Figs. 1b, 2). The diameters of cells supplemented at 1.5 mg/mL with whole homogenate were frequently over three times that of unsupplemented controls. Plasma supplementation was not associated with increased mean cell size. The proportion of tomons and cell clusters was maximal at the 0.75 mg/mL supplementation level in all treatments (Fig. 1c).

Unsupplemented cultures did not contain detectable numbers of tomons or clusters.

There were differences in the staining characteristics of the supplemented cultures. The cytoplasm of supplemented cells, particularly those which were in tomons or clusters, staining considerably more basophilic than did the control cultures (Fig. 2).

*Oyster tissue supplements differentially affect protease expression pattern and activity*

Supernatants from both the adductor muscle- and heart-supplemented cultures contained low molecular weight proteases (30-45 kDa), with highest activity at the lowest supplement concentration for the adductor muscle and at the highest concentration for the heart (Fig. 3). The zymograms of the gill/mantle and labial palp had a decreasing level of high molecular weight protease activity with increasing supplementation. Both had very faint low molecular weight protease bands at all levels of supplementation. The digestive gland/gonad and the whole oyster homogenates had decreasing protease activity with increasing supplementation, with the 1.5 mg/mL supplementation level of each having very little protease activity by zymogram. Both had very faint low molecular weight protease bands at the lowest supplementation levels only. Plasma-supplemented cultures had a protease expression pattern similar to unsupplemented control at low supplement levels, with a gradual diminishment in the intensity of digestion at higher supplementation levels, along with the appearance of faint low molecular weight protease bands. Unsupplemented cultures appeared to have the greatest high molecular weight, but no low molecular weight, protease activity.

The results of the quenched substrate protease activity assay did not consistently parallel the zymographic results. Unsupplemented cultures and cultures supplemented with 0.19 and 0.38 mg/mL plasma had similarly high activity (Fig. 3). Both the adductor muscle- and gill/mantle-supplemented cultures had decreasing protease levels with decreasing supplementation, though the gill/mantle-supplemented cultures had markedly higher activity. Labial palp- and digestive gland/gonad-supplemented cultures had very little protease activity at any supplementation level. This was also true of plasma-supplemented cultures at the 0.75 and 1.5 mg/mL supplement levels. Heart supplemented cultures had intermediate activity, with an increase in proteolysis at the lowest supplement level. Cultures supplemented with whole oyster homogenate had very low protease activity except at the highest supplement level. Unseeded supplemented media did not have any proteolytic activity (data not shown).

*Oyster tissue supplements differentially affect the quantity of p107 in the culture supernatants*

Culture supplementation decreased the level of p107 in the culture supernatant (Fig. 4). Unsupplemented cultures contained over 750 ng p107 per mL of medium. Regardless of supplement type, the level of p107 decreased with increasing supplement concentration up to 0.75 mg/mL. The p107 levels at 1.5 mg/mL were typically similar to those at the 0.75 mg/mL supplementation level. The p107 levels in plasma- and adductor muscle-supplemented cultures were least affected, though the level was reduced by nearly half with each twofold increase in supplementation from 0.19 to 0.75 mg/mL. Labial palp supplemented cultures had a similar pattern, but levels were lower than with plasma or adductor muscle. In all other supplemented cultures, p107 levels were greatly reduced, with 100 ng/mL or less of p107 in all treatments. Whole oyster-supplement caused the most significant change, with p107 levels nearly fifty times less than the unsupplemented control cultures at the highest supplementation levels.

Figure 1. Cell count, percentage tomonts and cell size in supplemented cultures.

Total cell count (a), mean cell diameter  $\pm$  standard deviation (b) , and percent of particles which were multicellular tomonts or adherent clusters (c) in *P. marinus* cultures supplemented with 1.5, 0.75, 0.38, or 0.19 mg/mL of supplements derived from oyster adductor muscle (Add), heart, gill and mantle (G/M), labial palps (LP), digestive gland and gonad (DG/G), whole body, or plasma. Duplicated cultures were seeded at  $10^6$  cells/mL in two mL of each medium and incubated for six weeks at 27°C in 5% CO<sub>2</sub> in a humidified incubator. Cells and tomonts were enumerated with a hemacytometer, and ten cells per treatment were sized by ocular micrometer. The heart supplement was not tested at the 1.5 mg/mL concentration due to an inadequate amount of material.

Figure 1.

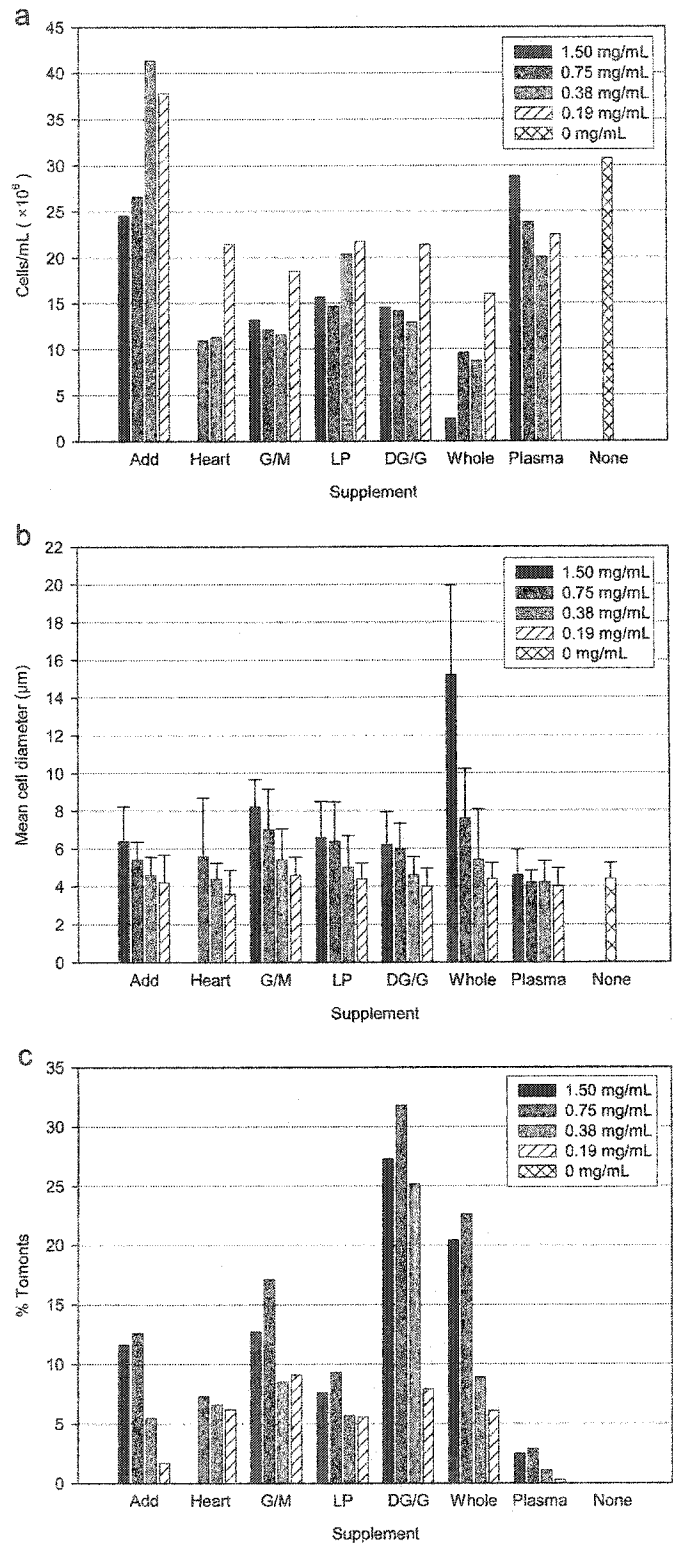


Figure 2. Light micrograph of a representative field of *P. marinus* cultures supplemented at 1.5 mg/mL, 0.75 mg/mL, 0.38 mg/mL, and 0.19 mg/ml, or unsupplemented demonstrating size, morphologic, and stage differences, including trophozoites (a, b), adherent cell clusters (c), tomonts (d), and enlarged, prezoosporangium-like trophozoites (e). Thirty three  $\mu$ L of each supplemented *P. marinus* culture was diluted to 200  $\mu$ L and applied to a glass slide by cytospin. The slides were air dried, methanol fixed, stained using Hema-3, and photographed on a phase contrast light microscope at 100X.

Figure 2.

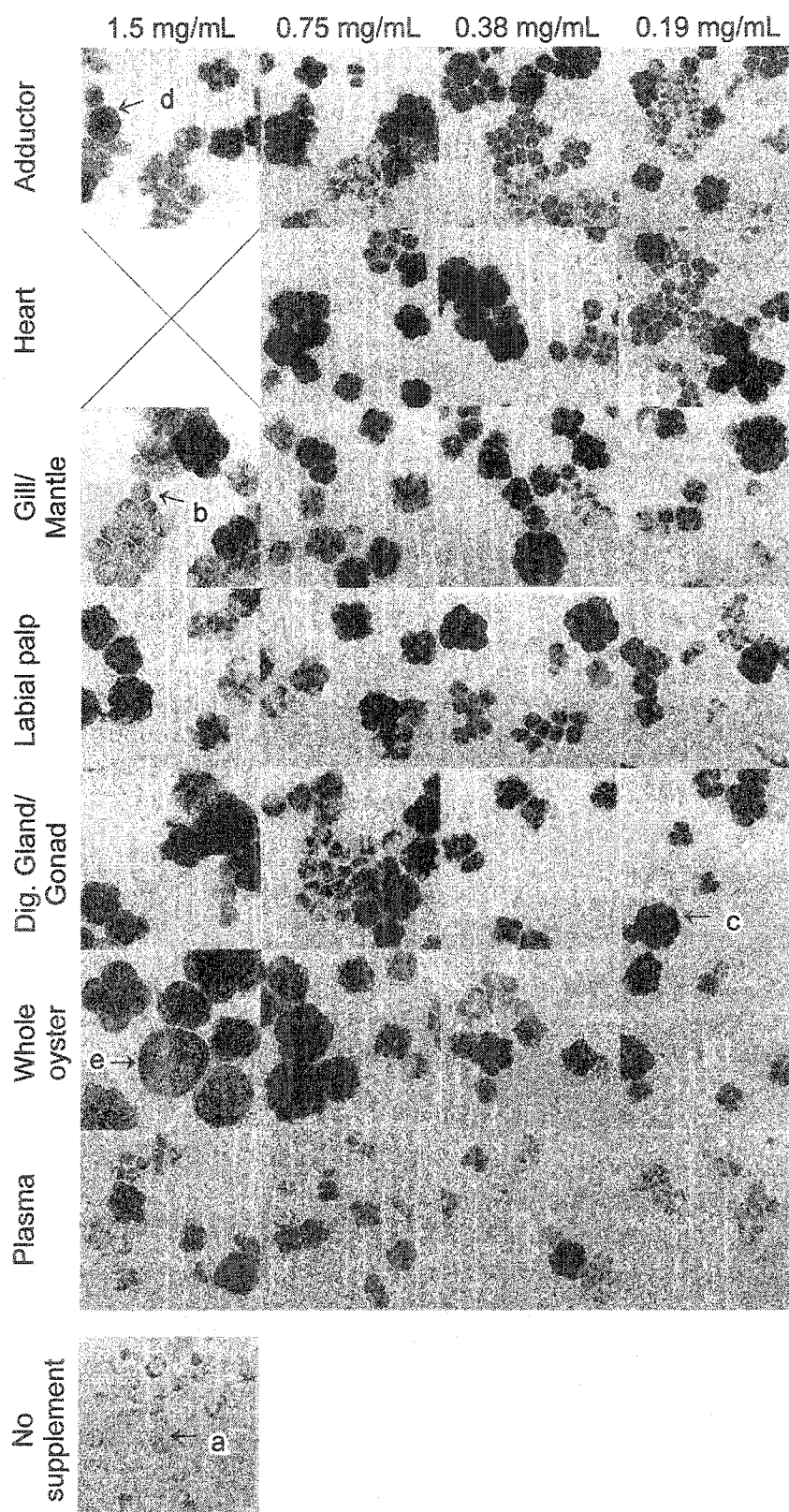


Figure 3. Gelatinase activity of six week supplemented *P. marinus* culture supernatants.

(a) Total gelatinase activity was measured by the rate of change in fluorescence of a quenched, fluoresceinated gelatin as a result of the proteolytic activity in 70  $\mu\text{L}$  of each culture supernatant combined with 10  $\mu\text{L}$  of buffer (0.5M Tris, 2mM  $\text{NaN}_3$ , 100mM EDTA) and 20  $\mu\text{L}$  of DQ gelatin substrate (1.0 mg/mL). Each supernatant was analyzed in duplicate.

SDS-PAGE zymograms (b) (8% acrylamide, 0.1% porcine gelatin) of 25  $\mu\text{L}$  of culture supernatant. Following electrophoresis, the gels were washed three times for ten minutes in 2.5% Triton-X-100, once in 0.1 M Tris pH 8.0, then incubated at 37°C in 0.1 M Tris, 10mM EDTA pH 8.0 for either one (short incubation) or sixteen (long incubation) hours. The gels were stained with Coomassie brilliant blue G-250.

Figure 3.

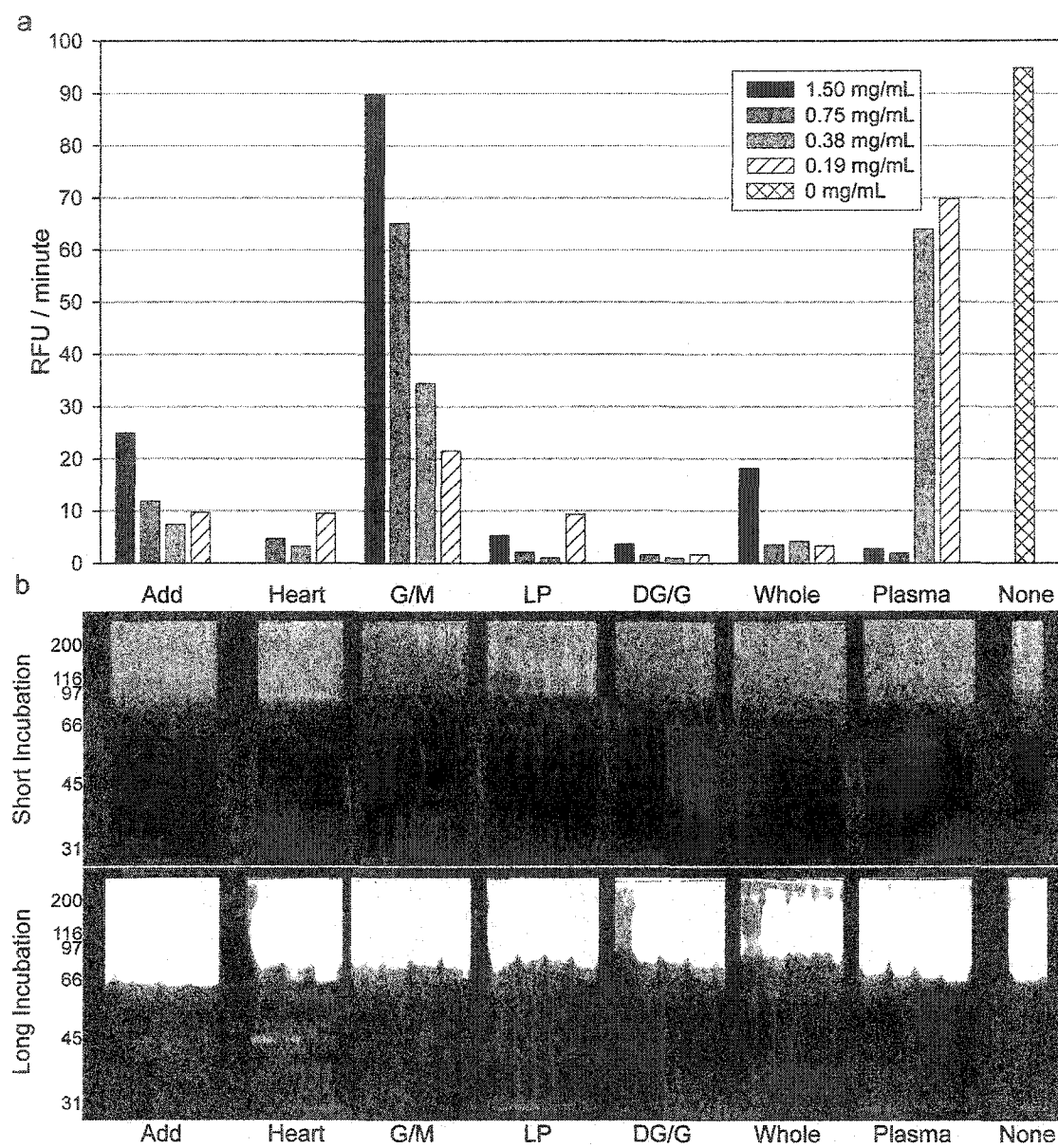
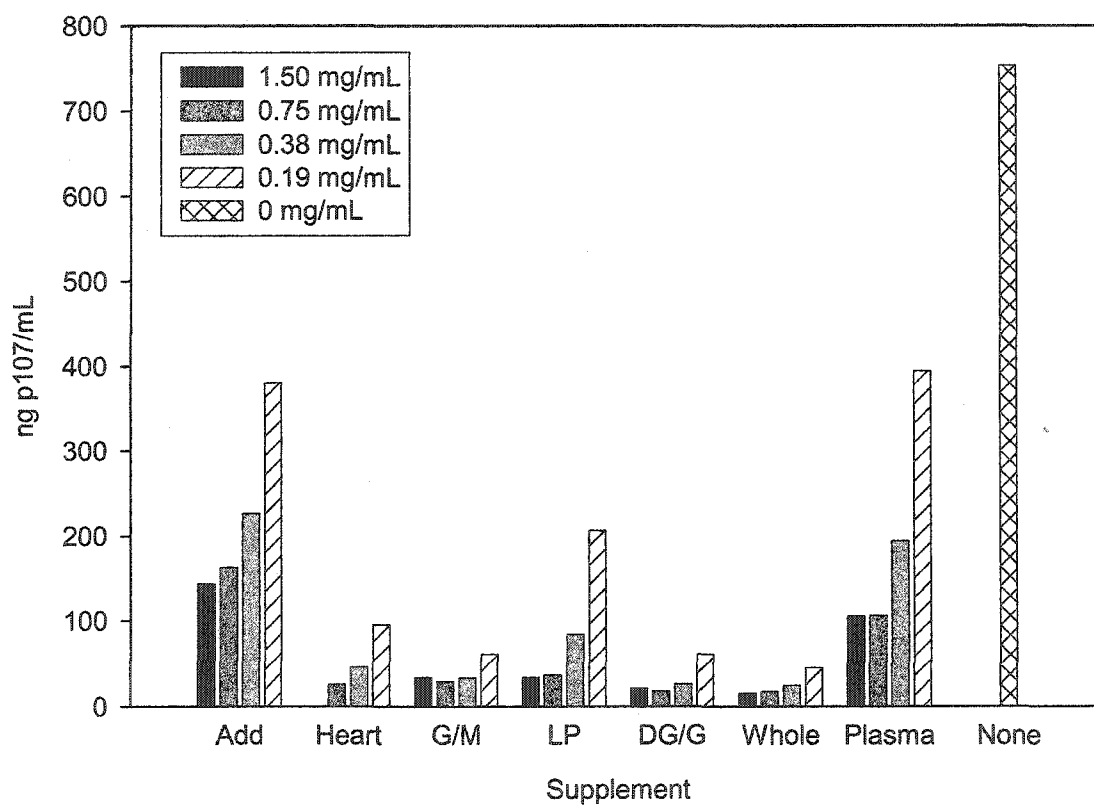




Figure 4. Quantification of p107 in culture supernatants

ELISA plates were coated with 200 ng of antibody 5G9 and blocked with 1% BSA in TTBS. Serial twofold dilutions of each culture supernatant sample were assessed in duplicate. The bound antigen was then detected by incubation with 200 ng/well of biotinylated antibody 1B3 followed by incubation with a 1:1000 dilution of peroxidase-conjugated streptavidin. The titrated rates of conversion of ABTS were compared and p107 was quantified by comparing the curves at 30% of the maximum rate plateau for the standard ECP (200 ng p107/mL) on that plate.

Figure 4.



## Discussion

The ability to selectively and independently modulate cell size, morphology, proliferation, and constituents ECP of this protozoan may offer tools for the investigation of modes of differentiation and virulence. In earlier studies, it was determined that addition of host-derived supplements to parasite cultures could modulate these phenotypic traits (MacIntyre et al., 2003; Earnhart et al., 2004). Further, supplementation also partially reversed the *in vitro* virulence attenuation that occurs quite rapidly in this organism (Ford et al., 2002; Earnhart et al., 2004). The interrelationships and induction modes of these various phenotypic changes were quite complex, and could not be separately studied using whole oyster-derived supplements. In this study, medium supplements created by homogenization of organs and tissues were capable of eliciting the multiple phenotypic changes observed with whole oyster homogenate supplements, including altered cell proliferation, size and morphology, and ECP composition.

Cell counts could be altered either positively or negatively, as compared with unsupplemented cultures, depending on the origin of the supplement. While nearly all of the supplements resulted in lower cell counts compared with unsupplemented control cultures, there was a striking increase in cell count that occurred with the lower concentrations of adductor muscle supplement (Fig. 1a), and none of the plasma supplemented cultures had the same degree of suppressed proliferation seen with other tissues. *In vivo*, *P. marinus* cells exist both within phagosomes of the hemocytes and free in oyster plasma (Perkins, 1996). The adductor muscle is highly vascular, and proliferation may be altered in plasma and adductor muscle supplemented cultures by similar mechanisms. In contrast, cultures supplemented with whole oyster homogenate showed very little proliferation. This confirms what has been previously described (Earnhart et al., 2004), and while it does not offer new insight into a more specific mechanism of suppression of replication, when combined with cell size data, it emphasizes the remarkable changes that can be elicited by high concentrations of whole

oyster homogenate supplement (Figs. 1a, 1b). The very large cells produced in these cultures are likely a partial transition to prezoosporangia (Figs. 1b, 2) (Earnhart et al., 2004; See this dissertation, Chapter 6). Differentiation into this stage has formerly been induced *in vitro* by prolonged incubation in a fluid thioglycollate medium (Ray, 1952; Ray, 1966; Choi et al., 1989; Bushek et al., 1994; Fisher and Oliver, 1996). Historically, transfer of these prezoosporangia to sea water resulted in maturation and release of motile zoospores (Perkins and Menzel, 1966). Over the past several years, for unknown reasons, *P. marinus* prezoosporangia induced by this technique have failed to zoosporulate (Kleinschuster et al., 1994; Perkins, 1996), though small numbers of zoospores have been produced using FBS supplemented medium (Sunila et al., 2001) and in thioglycollate supplemented with lipids (Chu and Lund, 2004). The production of zoospores *in vivo* is thought to occur in moribund oysters (Ford and Tripp, 1996), though this has not been well demonstrated. Culture supplementation with high doses of whole oyster homogenate, or possibly with higher doses of individual tissues than were tested in this study, may provide a means to investigate the inductive mechanism of cell enlargement into prezoosporangia, and possibly the mechanisms by which zoosporulation is triggered.

In addition to the changes in cell proliferation, supplementation resulted in reproduction by palintomy in tomons, rather than by the binary fission normally seen in unsupplemented cultures (Fig. 1c). Previous studies indicated that whole oyster supplement was an efficient inducer of this replicative form (MacIntyre et al., 2003; Earnhart et al., 2004); however, supplementation with homogenate of dissected digestive gland/gonad was even more effective. During active infection, *P. marinus* cells are frequently found, and often as tomons, in the connective tissues and epithelium of the gut, digestive gland, gill, and mantle, and in hemocyte phagosomes (Perkins, 1996). In this experiment, the gill/mantle- and digestive gland/gonad-supplemented media were found to have the largest proportion of tomons (Fig. 1c), possibly indicating a greater

concentration of an inducer of this replicative stage. The presence of replicative stages did not, however, predict the culture cell count. For example, despite the elevated number of cells found in plasma-supplemented cultures, these cultures had very few tomonts. While this could indicate that the cells rapidly dissociated following rupture of the tomont wall, it is more likely that the increase in cell counts in those cultures was due to binary division, as occurs in unsupplemented cultures. These results imply that parasite proliferation may be regulated by at least two factors.

Supplementation of cultures with tissue homogenates variably altered the pattern of protease secretion by *P. marinus* cells. The use of both gelatin zymography and a fluorogenic gelatin substrate allowed an understanding of the protease activity both in the presence of potential protease inhibitors and following their electrophoretic dissociation from the proteases, and also allowed quantification of protease activity not detectable by zymography. Zymographic evidence of elevated low molecular weight protease activity in the presence of heart and adductor muscle supplementation indicates the presence of inductive components in muscle tissue. Low molecular weight protease activity varied directly with heart supplementation, but indirectly with adductor muscle supplementation, indicating that the inducing factor may operate within a range bounded by the concentrations present in the two supplements. This dose-dependent induction of low molecular weight proteases has been reported from cultures supplemented with both whole oyster homogenate and oyster plasma (MacIntyre et al., 2003). The failure of whole oyster homogenate to induce low molecular weight proteases in this study indicates that the presence of these proteases is not simply due to specific induction by a certain tissue or molecule, but must also be co-regulated by other factors. This could involve protease destruction or inactivation by oyster proteases or inhibitory molecules (Oliver et al., 1999; Oliver et al., 2000; Ziegler et al., 2002; Muñoz et al., 2003). If the downregulation is due to inhibition it is likely not due to a reversible non-covalently bound inhibitor, as that type of inhibitor would be expected to dissociate from the

molecule during SDS treatment and/or electrophoresis. The possibility of protease destruction or inhibition is supported by the reduced high molecular weight protease activity in cultures supplemented with high levels of whole oyster or digestive gland/gonad homogenates. The reduction is similar in both cultures, and does not appear to be related to the cell count (Fig. 1a, 3). While the pattern of protease expression by *P. marinus* prior to experimental infection has not been demonstrated to be a reliable predictor of increased virulence (Earnhart et al., 2004), the regulation of a subset of proteases in the presence of a specific host tissue makes them an interesting target for further functional study. Alteration in protease expression has been demonstrated to occur in *Plasmodium falciparum* in association with erythrocyte invasion, hemoglobin degradation, processing of parasite proteins, and release of the parasite from the host cell (Lyon and Haynes, 1986; Rosenthal et al., 1987; Braun-Breton and Pereira da Silva, 1988; Greenbaum et al., 2002; Wickham et al., 2003).

The results of the fluorogenic gelatin assay indicate that there are likely multiple factors regulating total protease activity within the cultures. Plasma supplemented cultures appear to have inhibited proteolytic activity at the highest levels of supplementation. Protease inhibitors present in oyster plasma which are active against *P. marinus* proteases have been previously described (Faisal et al., 1998; Oliver et al., 1999; Oliver et al., 2000), so these findings are not altogether surprising. There was suppression of proteolytic activity in all other supplemented cultures at all doses, except those supplemented with gill/mantle. With increasing gill/mantle supplementation, there was a marked increase in rate of proteolysis, which was not reflected in the zymograms. Taken together, these results indicate that there may be induction of proteases not detectable zymographically, possibly in combination with inhibition or downregulation of the detectable proteases. The genome of the protozoan parasite *Entamoeba histolytica* contains at least 20 potentially functional cysteine protease genes; however, 90% of the protease activity found during *in vitro* culture is mediated by only three proteases

(Bruchhaus et al., 2003). *Perkinsus marinus* may also have the potential to secrete numerous proteases *in vivo*, or under specific culture conditions, of which only a subset may be detectable zymographically.

The extracellular product protein, p107, was found in all supplemented cultures, though in a wide range of concentrations. The general reduction in the p107 level with supplementation is in agreement with previous findings (See this dissertation, Chapter 6), and may indicate that various tissues are able to regulate the levels of this potential virulence factor to various degrees. The highest levels of p107 in supplemented cultures were with plasma and adductor muscle supplements, implying that the factor or factors involved in modulation of p107 expression may be present to a lesser degree in these supplements.

The varied physiological and morphological changes brought about by specific medium supplements may have some relevance to the *in vivo* disease process, and may conceivably represent requisite differentiative changes that must occur during normal infection. The large proliferative response seen in cells exposed to plasma and the vascular adductor muscle may, for example, parallel the initial rapid replication seen in early infection *in vivo* (Saunders et al., 1993). Similarly, the transition to prezoosporangia in the presence of whole oyster tissue homogenate may parallel that same transition seen *in vivo* in moribund oysters. That supplement may replicate conditions found in terminally infected oysters by releasing large amounts of tissue proteins, lipids, and enzymes, and by altering such medium conditions as the reduction-oxidation potential. While the alterations in protease and other protein production does not have a clear *in vivo* correlate, additional information regarding the function of these molecules may help elucidate the mechanism and purpose of their regulation under various medium conditions.

In addition to its importance as an oyster parasite, the phylogenetic position of *P. marinus* potentially makes it a useful model organism. *Perkinsus* occupies the proposed

phylum Perkinsozoa (Noren et al., 1999), within the alveolates (Cavalier-Smith, 1993), in a position apparently basal to the apicomplexans and dinoflagellates (Fong et al., 1993; Goggin and Barker, 1993; Siddall et al., 1995; Reece et al., 1997; Siddall et al., 1997). Culture techniques are not available for many of the alveolates, though where available, they have been an important tool for understanding nutritional requirements, metabolic pathways, virulence factors, and triggers for life stage differentiation. Culture of many of the alveolates requires use of a host cell line, such as with *Cryptosporidium* (Upton et al., 1995; Hijjawi et al., 2001; Arrowood, 2002; Hijjawi et al., 2002), *Toxoplasma* (Hughes et al., 1986; Chatterton et al., 2002; Sreekumar et al., 2003), *Babesia* (Levy and Ristic, 1980; Goff and Yunker, 1988; Schuster, 2002a), *Theileria* (Preston et al., 2001; Schuster, 2002a; Zweygarth et al., 2002), and *Plasmodium* (Trager and Jensen, 1997; Schuster, 2002b; Hurd et al., 2003). Few extracellular alveolate parasites are axenically culturable. These include the crustacean dinoflagellate parasite *Hematodinium* (Appleton and Vickerman, 1998) and the molluscan parasite *Perkinsus* (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; LaPeyre et al., 1993; Dungan and Hamilton, 1995; Gauthier et al., 1995; Gauthier and Vasta, 1995; LaPeyre and Faisal, 1995a; LaPeyre and Faisal, 1997). Given the relative ease of isolation and culture and the ability to manipulate virulence as well as several phenotypic traits by medium supplementation, *Perkinsus* represents a potentially useful organism in which to investigate shared metabolic, inductive, and pathogenic mechanisms of the dinoflagellates and apicomplexans.

In this study, marked changes in *P. marinus* were induced by supplements derived by homogenization of distinct oyster tissues. Morphological changes include enhanced proliferation in the absence of multicellular tomont structures by adductor muscle supplements, reproduction by palintomic fission by whole oyster digestive gland/gonad supplementation, and possible partial transition to the prezoosporangia stages at the highest levels of whole oyster supplementation. Biochemical changes include



downregulation of zymographic protease activity by supplemented cultures, but with evidence of induction of proteolytic activity by gill/mantle which is not evident on the zymograms, as well as upregulation of low molecular weight proteases by supplementation with homogenized heart or adductor muscle, and alteration of the concentration of a protein extracellular product, p107. This fractionation method was capable of inducing marked changes in this parasite with surprising specificity. Further refinement of these supplements may allow a better understanding of this parasite as well as potential shared regulatory mechanisms of the alveolates.

## Chapter 4

### **The humoral response to *in vitro* generated parasite antigens is enhanced by the removal of a defined media component prior to immunization**

#### **Abstract**

Concentrated culture supernatants containing the extracellular products (ECP) of the protozoan oyster parasite *Perkinsus marinus* were used to immunize mice. This preparation, produced by ultrafiltration, was found to be both poorly immunogenic and toxic to experimental animals. The possibility that these effects were due to toxic parasite products and/or medium constituents was examined. Co-administration of this material with highly immunogenic oyster hemolymph caused a substantive suppression of the specific antibody response to hemolymph, as well as a decrease in the number of epitopes recognized. Potential protein/protease toxin-mediated causes of the immunosuppression were addressed by heat denaturation and proteolytic inhibition of the concentrate; neither substantially enhanced immunogenicity. Analysis of media constituents revealed that the known immunomodulatory surfactant, Pluronic F-68 (PF68), used in the defined lipid concentrate supplement, was capable of eliciting significant immunosuppression. Although isolated protein antigens from *P. marinus* ECP remain highly immunosuppressive, separation of the protein antigens from the PF68 has enabled production of polyclonal antisera with a broader recognition of antigens.

## Introduction

Extracellular products (ECP) elaborated by parasites amenable to *in vitro* culture can be of particular value in the study of potential virulence factors, enabling, for example, the production of highly useful and specific poly- and monoclonal antibodies (Bates et al., 1987; Langer et al., 2002). The oyster parasite *Perkinsus marinus* is an axenically culturable protozoan that causes a chronic wasting disease in the eastern oyster, *Crassostrea virginica*, and has been responsible for much of the decline in that fishery along the eastern seaboard of the United States (Burreson and Ragone-Calvo, 1996; Ford, 1996). *Perkinsus marinus* secretes multiple ECP into the culture medium, and thereby provides an excellent model to investigate the mechanisms of protozoan virulence. Several medium formulations containing a variety of supplements including yeastolate, fetuin, fetal bovine serum, bovine serum albumin, and oyster hemolymph and tissue homogenates have been employed in *P. marinus* culture (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; LaPeyre et al., 1993; Dungan and Hamilton, 1995; Gauthier et al., 1995; LaPeyre and Faisal, 1997; MacIntyre et al., 2003). The expression profile of the ECP can be selectively altered by these media constituents, allowing a greater ability to study regulation of potential secreted virulence factors, including several serine proteases (LaPeyre and Faisal, 1995b; MacIntyre et al., 2003). Chemically defined and protein-free media are of particular value in the production of antisera against potential extracellular virulence factors, as they eliminate the need to include potentially immunogenic non-parasite proteins along with the parasite antigens during immunization. *Perkinsus marinus* can be cultured in such a defined medium, ODRP-3, which is made from a base containing commercially available amino acid, vitamin, and defined lipid supplements (LaPeyre and Faisal, 1997). ECP are harvested from these media supernatants and are typically concentrated by ultrafiltration (LaPeyre et al., 1995; Garreis et al., 1996; LaPeyre et al., 1996; Oliver et al., 1999; Oliver et al., 2000).

Attempts have been made to produce antisera against *P. marinus* cells and/or ECP in both rabbits (Choi et al., 1991; Dungan and Roberson, 1993; Ottinger et al., 2001) and mice (Dungan and Roberson, 1993); however, such immunizations have typically induced poor titers and a predominant IgM response. In addition, we have observed that immunization of mice with *P. marinus* ECP is associated with significant toxicity. Mice immunized with ECP in complete Freund's adjuvant (CFA) succumbed within sixteen hours of immunization, apparently as a result of hemorrhage. Immunization with Freund's incomplete adjuvant (FIA), while associated with less toxicity, generates a very poor immune response. Thus, determination of the cause of ECP toxicity and immunosuppression were viewed as crucial problems requiring resolution before further progress could be made toward production of antibodies useful for the study of parasite virulence.

## Materials and Methods

### *Animals*

Six-week-old female BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were cared for in accordance with the 1996 National Research Council Guidelines for the Care and Use of Laboratory Animals. All immunizations employed a 1:1 emulsion of antigen with either CFA or FIA.

### *In vitro propagation of Perkinsus marinus and production of ECP*

*Perkinsus marinus* isolate P-1 (LaPeyre et al., 1993) was seeded at  $10^6$  cells per mL in 50 mL of ODRP-3 media in a 75cm<sup>2</sup> tissue culture flask and grown at 27°C under 5% CO<sub>2</sub> for six weeks. The cells were removed from the culture by centrifugation and the supernatant passed through a 0.22µm filter to remove any residual cells and debris. The culture supernatant was placed into 10kD molecular weight cutoff ultrafiltration concentrators (Centriprep-10, Millipore, Bedford, MA) and concentrated tenfold by

centrifugation at 2500 x g at 4°C. The buffer was exchanged by suspension of the ECP in phosphate buffered saline (100mM phosphate, 150mM NaCl, pH 7.4) and re-concentration in the same Centriprep-10. The resultant culture supernatant ECP preparations (S-ECP) were stored at -80°C.

#### *Analysis of antibody titers*

Assessment of the humoral response to hemolymph or ECP preparations was by enzyme-linked immunosorbant assay (ELISA). High protein binding ELISA plates (Costar 3590, Corning, Inc., Corning, NY) were coated with 25 to 100ng of antigen per well in 50µL of carbonate buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 17.4mM NaHCO<sub>3</sub>, pH 9.7), and residual unbound sites were blocked with 240µL/well of 1% bovine serum albumin (BSA) in Tris buffered saline with Tween-20 (TTBS; 50mM Tris, 150mM NaCl, 1mM EDTA, 0.1% Tween-20, pH 7.5). Fifty µL of serial 1:5 dilutions of a 1:50 dilution of mouse serum in 1% BSA in TTBS were then assayed in duplicate. The bound IgG was detected using 50µL of a dilution of peroxidase-labeled Fc-specific goat-anti-mouse IgG secondary antibody (#115-035-008, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and quantified by colorimetric detection of the rate of conversion of 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS). Titers were calculated as the dilution corresponding to 50% of the maximum rate plateau (Butler, 1994).

#### *Immunization with heat or phenylmethanesulfonyl fluoride treated S-ECP*

As *P. marinus* ECP contain a high level of serine protease activity (LaPeyre and Faisal, 1995b; LaPeyre et al., 1995), inactivation of such activity by heat or the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was conducted to determine if this inactivation would decrease toxicity or enhance immunogenicity. S-ECP were heat-inactivated by incubation for 3 minutes in a boiling water bath. Serine protease inactivation was accomplished by incubation in 2mM PMSF. The unreacted PMSF was

allowed to degrade through approximately twenty half-lives prior to immunization (James, 1978). One mouse per treatment was immunized with 50 $\mu$ g of untreated S-ECP, heat-treated S-ECP or PMSF-treated S-ECP in FIA in divided intraperitoneal and subcutaneous depots. The anti-S-ECP response was analyzed by ELISA at four weeks post-immunization as described above. ELISA wells were coated with 100ng S-ECP, and secondary antibody was applied at a 1:1000 dilution.

Western blots were used to determine the pattern of reactivity in each of the mouse sera. Twenty  $\mu$ g of untreated S-ECP were separated under non-reducing conditions by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970). Molecular weights were estimated using biotinylated broad-range molecular weight markers (Bio-Rad Laboratories, Hercules, CA). The separated proteins were blotted onto polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) in tris-glycine-methanol buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3), and the blot was blocked with TTBS and cut into sections. Individual sections were probed with sera from mice immunized four weeks prior with untreated S-ECP, heat-treated S-ECP, or PMSF-treated S-ECP, at a dilution of 1:500. Secondary detection was by a peroxidase conjugated Fc-specific goat-anti-mouse IgG at a 1:10000 dilution. Markers were detected with a 1:3000 dilution of peroxidase-conjugated streptavidin (Sigma, St. Louis, MO). Reactive bands were visualized by film (Kodak Biomax MR-1) exposure using enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Piscataway, NJ), and the blot was stained for total protein using colloidal gold (Bio-Rad Laboratories, Hercules, CA).

#### *Assessment of immunosuppression by S-ECP*

To ascertain whether the lack of a humoral response to the complete array of S-ECP was due to immunosuppression, the impact of S-ECP on the antibody response to an

equally diverse, yet immunogenic set of proteins, oyster hemolymph, was determined. Three mice per group were immunized with 50µg of cell-free oyster hemolymph from *P. marinus* negative oysters, 50µg of S-ECP, or 50µg of S-ECP co-emulsified with 50µg of hemolymph in FIA in divided intraperitoneal and subcutaneous depots. Anti-hemolymph titers were assayed by ELISA at weeks four and eight post immunization as described above. ELISA wells were coated with 25 ng of oyster hemolymph protein, and secondary antibody was applied at a 1:5000 dilution. Anti-S-ECP titers were similarly assayed, with S-ECP coated at 50ng/well, and using a 1:1000 dilution of secondary antibody. At week 10, all mice, as well as three naïve mice, were immunized with 50µg of hemolymph in FIA. Four weeks later, anti-hemolymph titers were retested on all mice in a manner identical to that described above.

#### *Separation of Pluronic F-68 from ECP protein antigens*

Pluronic F-68 (PF68), along with other media components, were removed from potential protein antigens by ion exchange chromatography using DEAE-coupled Sepharose CL-6B equilibrated in 20mM piperazine (pH 9.7). An aliquot of S-ECP was extensively dialyzed against distilled/deionized water at 4°C using 10kDa molecular weight cutoff dialysis tubing. The dialyzed S-ECP were then mixed 1:1 with 40mM piperazine pH 9.7 and applied to the column at 2mL/min. The column was washed with five column volumes of piperazine buffer, and the bound ECP components were eluted using piperazine buffer with 2M NaCl. The eluate was extensively dialyzed against distilled/deionized water and concentrated in the dialysis tubing by reverse osmosis using polyethylene glycol.

#### *Immunization of mice with DEAE-purified ECP (D-ECP)*

Initially, a single mouse was immunized with 50µg of DEAE-purified ECP (D-ECP) emulsified in CFA, and closely monitored for toxic effects. This mouse was

subsequently boosted with 20 $\mu$ g at four weeks and 10 $\mu$ g at eight weeks in FIA. After confirmation of a lack of toxicity in the initial mouse, two additional mice per group were immunized with 100 $\mu$ g, 50 $\mu$ g, or 25 $\mu$ g of D-ECP in CFA and boosted after four weeks with either 10 or 20 $\mu$ g of D-ECP in FIA. Anti-D-ECP response was titrated by ELISA, as described above, after the second boost for the initial test mouse and after the first boost for all other mice. ELISA wells were coated with 50ng D-ECP and secondary antibody was applied at a 1:1000 dilution. Western blots against D-ECP were also completed as described above, with the sera tested at a 1:200 dilution using a Surf-blot apparatus (Idea Scientific Company, Minneapolis, MN).

*Isolation and assessment of immunomodulatory media components*

Hemolymph (25 $\mu$ g/mL) from *P. marinus*-free oysters was spiked with each of four media treatments. Treatments included complete ODRP-3 medium, ODRP-3 medium excluding the commercial defined lipid supplement (lipid-free medium), lipid-free medium with 22 $\mu$ g/mL Tween-80, or lipid-free medium with 1mg/mL Pluronic F-68 (PF68). The concentrations of Tween-80 and PF68 mimicked their respective concentrations in the complete media containing the commercial defined lipid mix. All of the above were then concentrated tenfold in 10kDa molecular weight cutoff Centriprep ultrafiltration devices following the protocol for S-ECP preparation (above). Three mice per group were immunized with 50 $\mu$ g of hemolymph proteins from each of the treatments and controls. Controls included 50 $\mu$ g of untreated hemolymph, 50 $\mu$ g hemolymph combined with 50 $\mu$ g D-ECP and 50 $\mu$ g hemolymph combined with 50 $\mu$ g BSA. All immunizations were administered in FIA in divided subcutaneous and intraperitoneal depots. Anti-hemolymph ELISA reactivity was titrated at four and eight weeks after immunization according to the method described above, with the exception that the plates were blocked with TTBS without BSA.



### *Quantification of Pluronic F-68 in ECP preparations*

Complete ODRP-3 medium was made excluding phenol red, due to its potential interference in the colorimetric assay. As with standard cultures, *Perkinsus marinus* was grown in the medium for six weeks prior to harvest of the culture supernatant. The culture supernatant was divided and processed in parallel by ultrafiltration concentration and DEAE column purification. PF68 concentration was estimated using a modification of the methods of Ghebeh et al. (1998). A standard curve of PF68 was created using dilutions of an aqueous solution of PF68, spanning concentrations from 0.1 to 2.0 mg/mL. Two hundred  $\mu$ L of each standard, test sample, or 1:10 dilution of test sample were placed into triplicate 1.6 mL microfuge tubes. One hundred  $\mu$ L of cobalt thiocyanate (3% cobalt nitrate, 20% ammonium thiocyanate in distilled/deionized water), 200 $\mu$ L of ethyl acetate, and 80 $\mu$ L of absolute ethanol were added to each tube. The tubes were then vortexed and centrifuged at 10000 g for 60 seconds. The upper ethyl acetate and middle aqueous layers were removed, and the sediment and tube walls were rinsed twice with 200 $\mu$ L of ethyl acetate. The sediment was then dissolved in 2 mL of acetone. The absorbance of all test and standard tubes was measured spectrophotometrically against an acetone blank at an absorbance of 328nm, and the concentration of PF68 was determined by interpolation to a least squares fit of the standard curve.

## **Results**

### *Heat and PMSF treatments do not substantially enhance S-ECP immunogenicity*

No anti-S-ECP titer could be calculated by ELISA in sera from mice immunized with untreated, heat-treated, or PMSF-treated S-ECP. There was a rate of change in the absorbance that was slightly higher than background at the highest concentrations of the serum produced against PMSF-treated S-ECP (Fig. 1A). By western blot, the serum against untreated S-ECP did not appear to detect any proteins despite extended film exposure. Serum against heat-treated S-ECP recognized seven bands, however the

reactivity was very weak. Serum against PMSF-treated S-ECP predominantly recognized a single band, with weak recognition of two other bands (Fig. 1B).

#### *S-ECP suppresses the IgG response*

The admixture of S-ECP with hemolymph caused a suppression of the anti-hemolymph humoral response (Figs. 2A, 2B). Mean anti-hemolymph IgG titers of sera from mice immunized with both S-ECP and hemolymph were approximately 325 times lower at both four weeks and eight weeks than sera from mice administered hemolymph alone. The anti-S-ECP titers in sera from mice immunized with S-ECP alone or S-ECP with oyster hemolymph were unmeasurable, with reaction rates near zero at all concentrations. Thus, the presence of oyster hemolymph did not have any adjuvant effect or otherwise enhance the immune response to S-ECP. Following a week 10 hemolymph reimmunization of all three groups plus immunization of a naïve group, the mean titer of sera from mice immunized with hemolymph and S-ECP was found to be similar to sera from mice immunized with hemolymph alone (Figs. 3A, 3B). Sera from naïve mice had a mean anti-hemolymph titer nearly twice that of mice immunized with S-ECP ten weeks prior to hemolymph exposure (Figs. 3A, 3B).

#### *The presence of Pluronic F-68 or media additives suppresses the IgG response*

In an attempt to ascertain whether PF68 or other components may contribute to immunosuppression, mice were immunized with hemolymph concentrated with ODRP-3 medium, lipid-free medium, lipid-free medium with PF68, lipid-free medium with Tween-80, or ECP depleted of media components by ion exchange chromatography (D-ECP). Ion exchange purification of ECP on DEAE-Sephacrose CL-6B did not visually alter the protein profile as visualized by SDS-PAGE (data not shown), but removed essentially all of the PF68, while ultrafiltration retentates (S-ECP) had significantly concentrated surfactant (Table 1). The mean anti-hemolymph IgG response was

diminished at weeks four and eight in the groups immunized with antigen in the presence of lipid-containing media, PF68, or D-ECP (Figs. 4A, 4B). In addition to the above groups, the mean titer in the group immunized with antigen spiked with lipid-free medium was reduced at week eight (Fig. 4B).

#### *DEAE-purified ECP has enhanced immunogenicity*

Although D-ECP appears to be independently immunosuppressive to heterologous antigens, as evidenced by the suppression of the antibody response to hemolymph antigens, it was nonetheless more immunogenic than S-ECP. This may have been due to the ability to administer the D-ECP in the presence of CFA without any overt toxicity. In all D-ECP immunized mice, anti-D-ECP titers were greater than the best titer achieved using S-ECP (Fig. 5A). The number of proteins recognized and the intensity of recognition by the sera generated against D-ECP was markedly improved over that generated by S-ECP, with recognition of a majority of the ECP proteins visualized by colloidal gold protein stain (Fig. 5B). There was no apparent association between increased levels of immunogen in the primary (i.e. 25, 50 or 100 $\mu$ g) or booster (i.e. 10 or 20 $\mu$ g) immunizations and the titer at week eight.

#### **Discussion**

Production of polyclonal antisera or a panel of monoclonal antibodies which detect distinct ECP components generated *in vitro*, can facilitate the study of the mechanisms of induction and secretion of potential virulence factors. Attempts to produce such tools for the study of *P. marinus* ECP have met with little success, primarily due to failure of the animals to mount an adequate immune response under immunization conditions that do not produce acute morbidity or mortality. The initial attempts to rectify this problem were aimed at general inactivation of potentially toxic molecules by heat treatment or inactivation of proteases (Fig. 1). These attempts were

Figure 1.

- A. ELISA titration of week four sera from individual mice immunized with 50 $\mu$ g S-ECP (■), 50 $\mu$ g heat-treated S-ECP (▲), or 50 $\mu$ g PMSF treated S-ECP (●) in FIA. ELISA wells were coated with 100ng of S-ECP and blocked with 1% BSA in TTBS. Each serum was serially diluted (1:5) in 1% BSA in TTBS and assayed in duplicate. Secondary antibody was a 1:1000 dilution of peroxidase-labeled goat-anti-mouse IgG.
- B. Western blot of 20 $\mu$ g of untreated S-ECP using a 1:500 dilution of sera from mice immunized with untreated, heat-treated and PMSF-treated S-ECP. Secondary detection was by a 1:10000 dilution of peroxidase-labeled goat-anti-mouse IgG. Total protein stained with colloidal gold and enhanced chemiluminescence immunoblots of serum from mice immunized with 50 $\mu$ g S-ECP, 50 $\mu$ g heat-treated S-ECP, or 50 $\mu$ g PMSF treated S-ECP. Molecular weights (left) are in kDa.

Figure 1A.

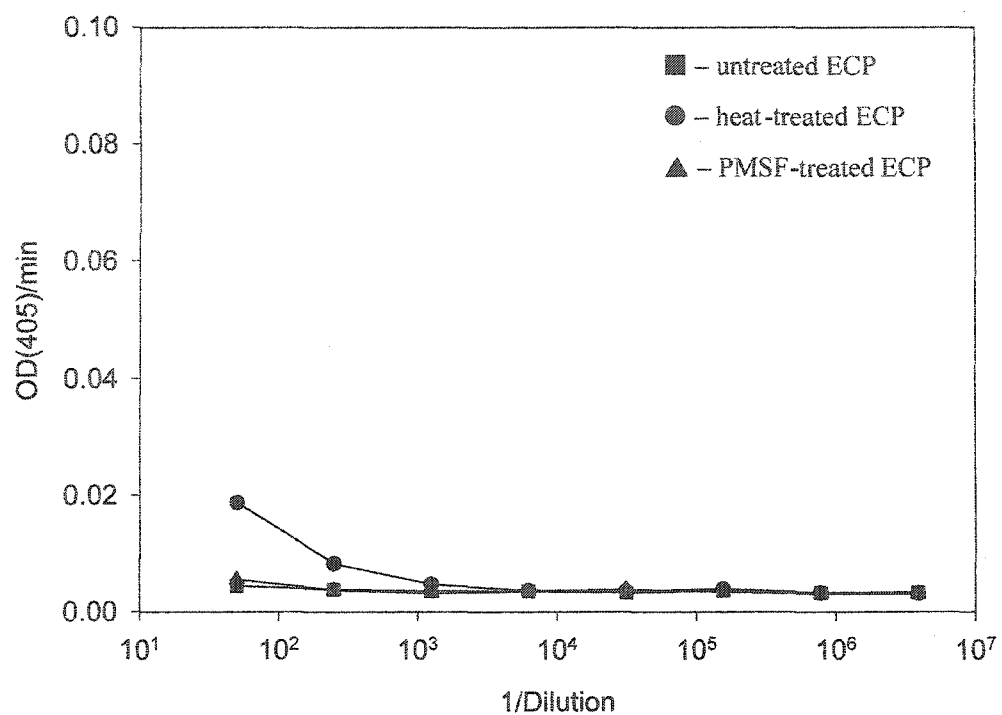


Figure 1B.

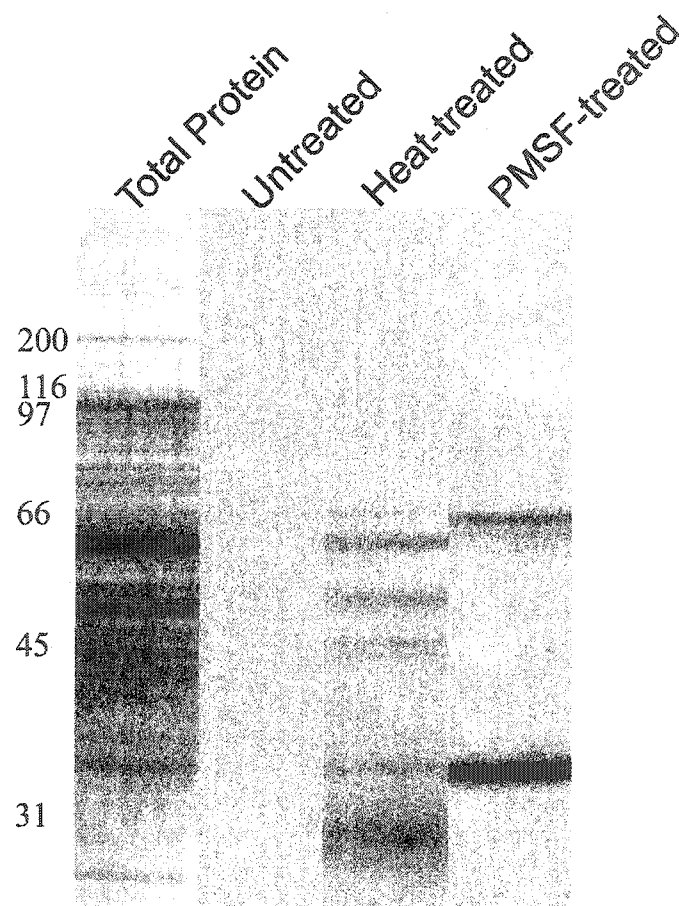


Figure 2.

ELISA titration of week four (A) and week eight (B) IgG-specific anti-hemolymph response of mice (three per treatment) immunized with S-ECP (●), S-ECP + hemolymph (▲), or hemolymph alone (■). ELISA wells were coated with 25ng of hemolymph and blocked with 1% BSA in TTBS. Each serum was serially diluted (1:5) in 1% BSA in TTBS and assayed in duplicate. Secondary antibody was a 1:5000 dilution of peroxidase-labeled goat-anti-mouse IgG.

The maximum reaction rates of 0.13 OD(405)/min (week four) and 0.135 OD(405)/min (week eight) used to calculate titer are marked by dotted lines. The titer was calculated as the inverse of the dilution corresponding to 50% of the maximum reaction rate plateau.

Figure 2A.

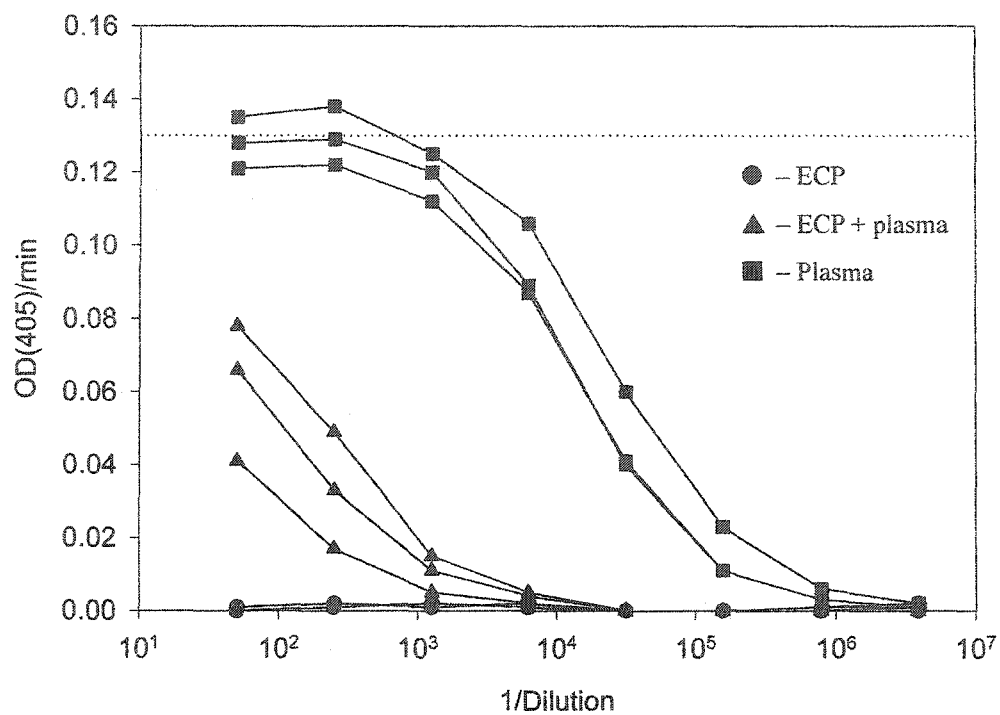


Figure 2B.

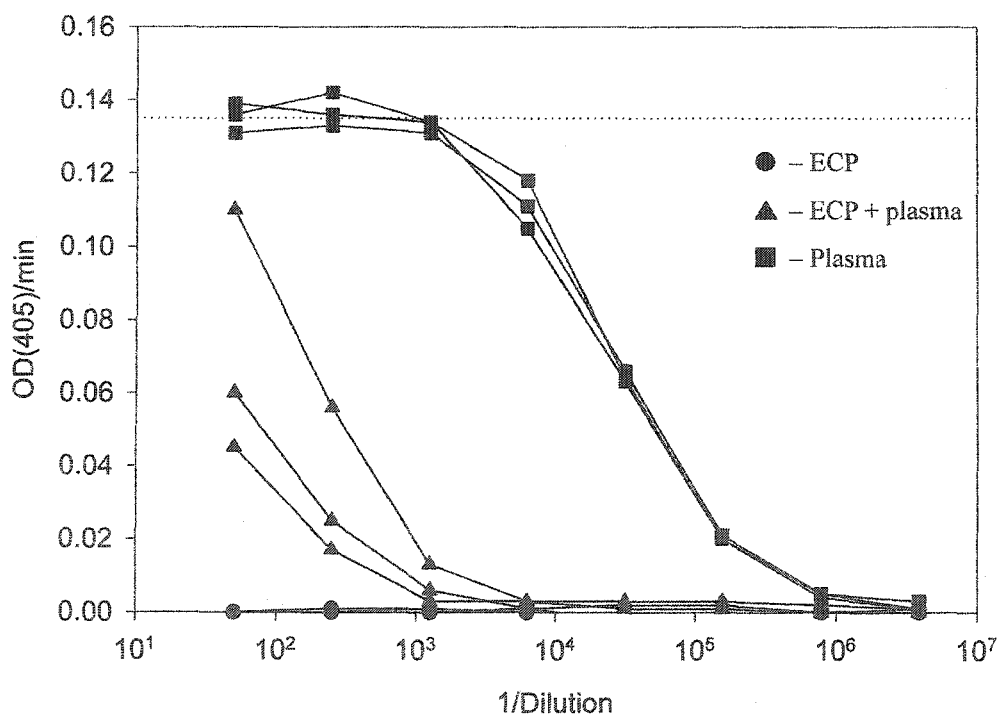


Figure 3.

- A. ELISA titration of week 14 IgG-specific anti-hemolymph response after week 10 hemolymph challenge of mice previously immunized with S-ECP (○), S-ECP + hemolymph (▲), hemolymph alone (■), or primary immunization of naïve mice (◇). ELISA wells were coated with 25ng of hemolymph and blocked with 1% BSA in TTBS. Each serum was serially diluted (1:5) in 1% BSA in TTBS and assayed in duplicate. Secondary antibody was a 1:5000 dilution of peroxidase-labeled goat-anti-mouse IgG. The maximum reaction rates of 0.17 OD(405)/min for mice initially immunized with hemolymph and for naïve mice, and 0.13 OD(405)/min for mice initially immunized with S-ECP + Hemolymph and S-ECP alone used to calculate titer are marked by dashed and dotted lines, respectively. The titer was calculated as the inverse of the dilution corresponding to 50% of the maximum reaction rate plateau.
- B. Calculated (○) and mean (+) titers of week 14 IgG-specific anti-hemolymph primary or memory response in mice initially immunized with hemolymph, S-ECP + hemolymph, S-ECP, or naïve mice.



Figure 3A.

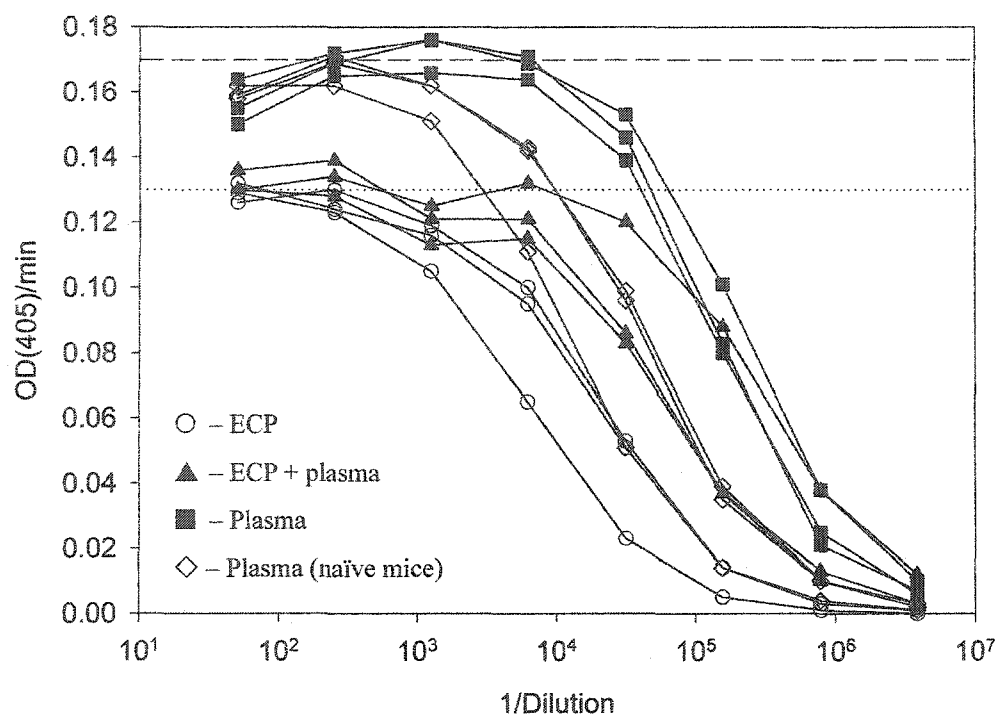
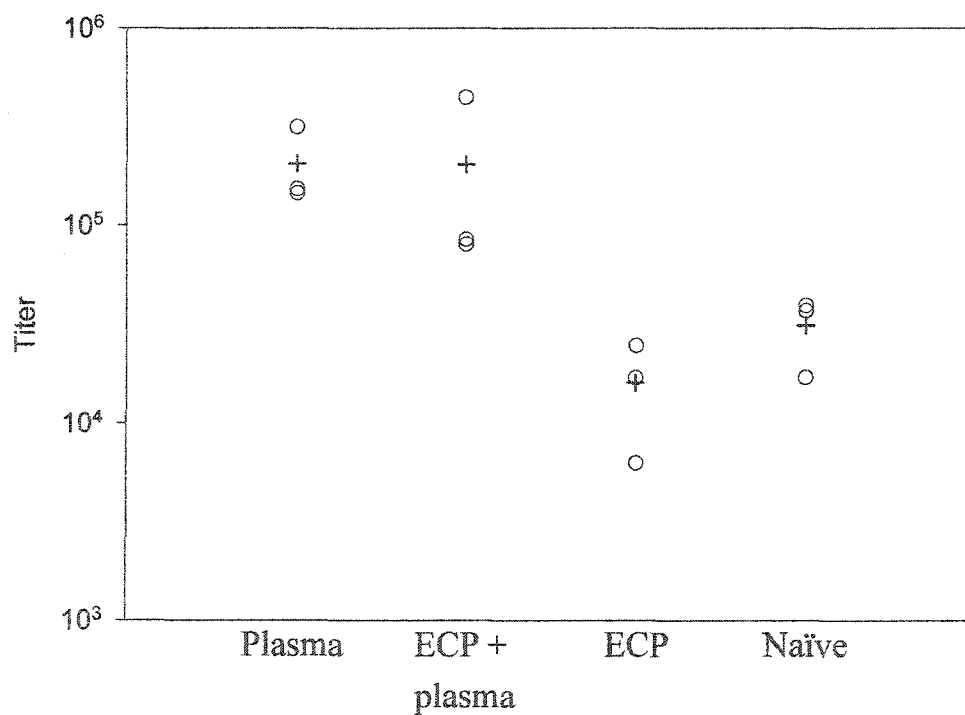


Figure 3B.



| Sample                                  | PF68 concentration (mg/mL) |
|---|----------------------------|
| ODRP-3 Medium                           | 0.92                       |
| Unprocessed culture supernatant         | 1.13                       |
| Pre-DEAE dialyzed supernatant           | 0.88                       |
| Final DEAE ECP (D-ECP)                  | <0.1                       |
| Ultrafiltration filtrate                | <0.1                       |
| Final ultrafiltration retentate (S-ECP) | 6.50                       |

Table 1. Concentration of PF68 in ODRP-3 medium, culture supernatants and ECP preparations. S-ECP and D-ECP were brought to the same volume prior to PF68 quantification.

Figure 4.

Calculated ELISA titers (mean  $\pm$  standard error) of week four (A) and week eight (B) IgG-specific anti-hemolymph response in the presence of no supplement, BSA, lipid-free medium, ODRP-3 medium, Tween-80, PF68, or D-ECP. ELISA wells were coated with 25ng of hemolymph and blocked with TTBS. Each serum was serially diluted (1:5) in TTBS and assayed in duplicate. Secondary antibody was a 1:5000 dilution of peroxidase-labeled goat-anti-mouse IgG. The titer was calculated as the inverse of the dilution corresponding to 50% of the maximum reaction rate plateau.

Note: One mouse in the D-ECP plus hemolymph group died on the second day after immunization, likely due to complications secondary to the injection.

Figure 4A.

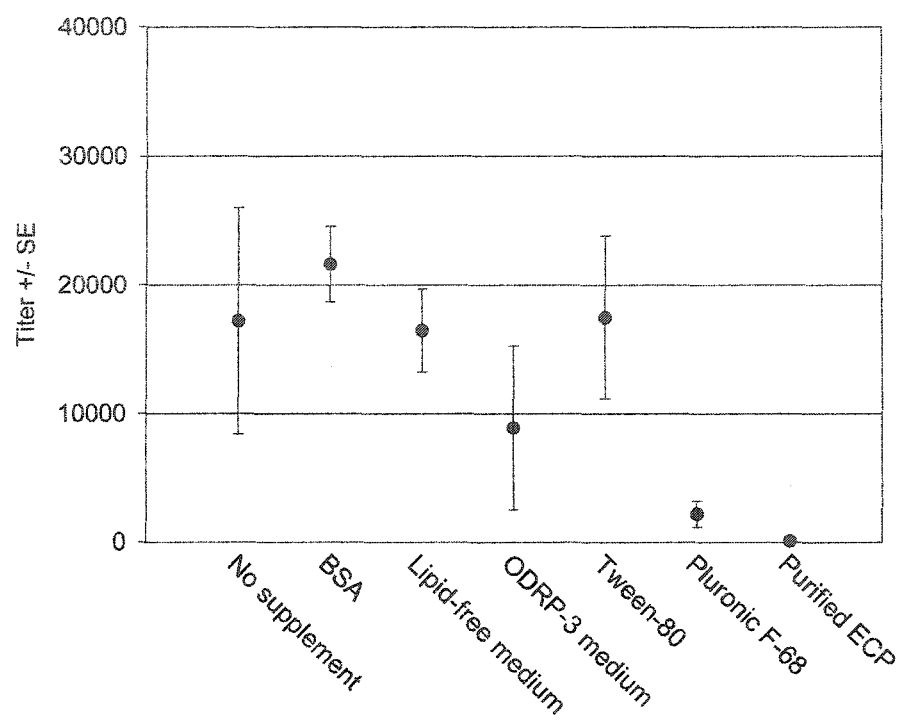


Figure 4B.

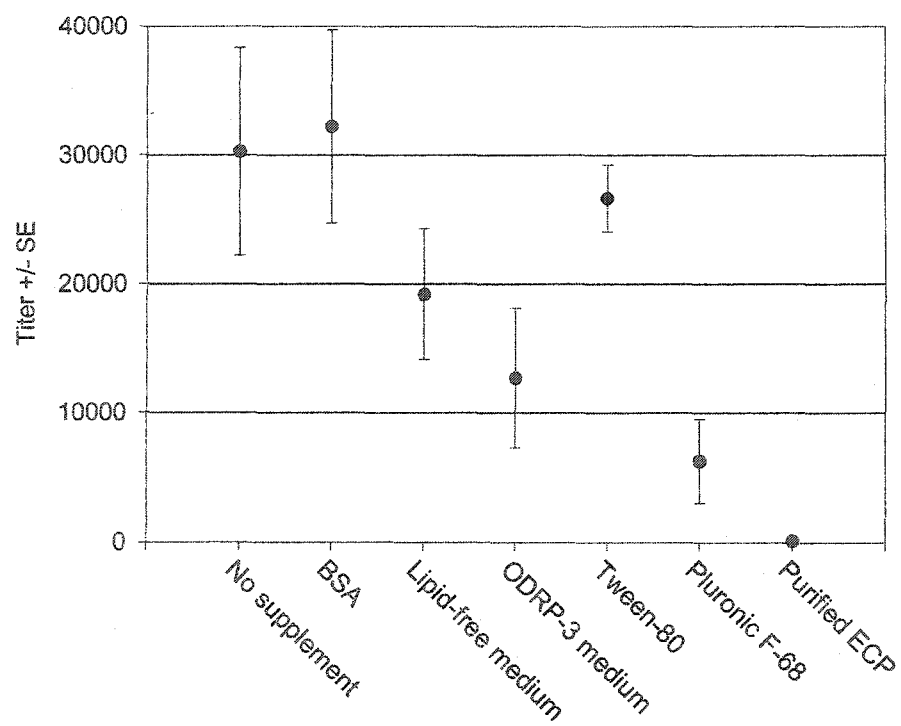


Figure 5A.

ELISA titration of sera from mice immunized with D-ECP, four weeks after final boost. ELISA wells were coated with 50ng of D-ECP and blocked with 1% BSA in TTBS. Each serum was serially diluted (1:5) in 1% BSA in TTBS and assayed in duplicate. Secondary antibody was a 1:1000 dilution of peroxidase-labeled goat-anti-mouse IgG. The immunization schedule for the mice is shown in the inset table.

\* - These sera (+) are from animals immunized with S-ECP FIA, and are included for comparison.

Figure 5A.

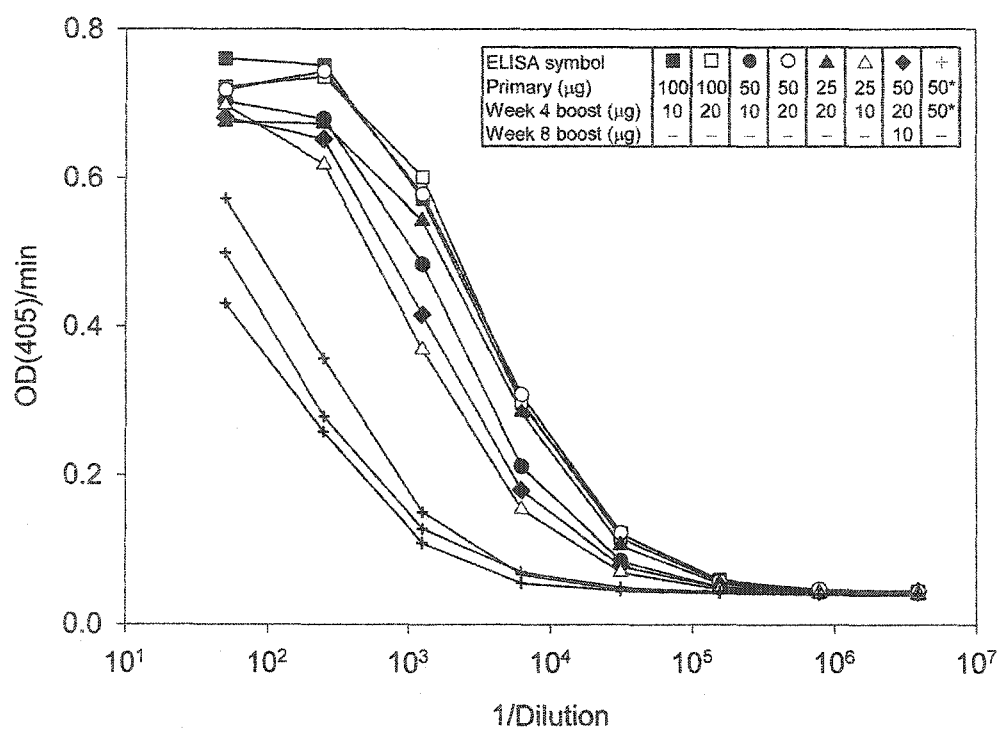
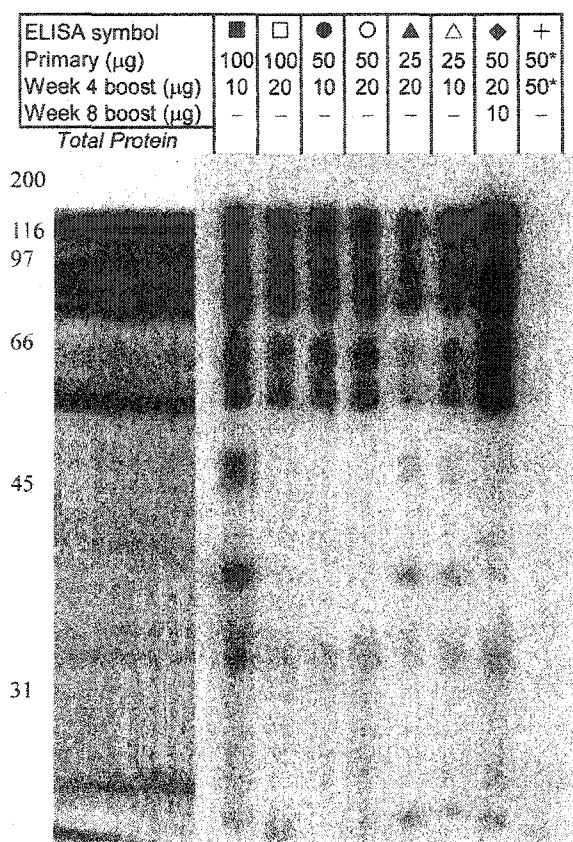


Figure 5B.

Western blot of 20µg of D-ECP using 1:200 dilutions of sera from mice immunized with D-ECP or S-ECP. Secondary detection was by a 1:10000 dilution of peroxidase-labeled goat-anti-mouse IgG. Colloidal gold total protein stain and enhanced chemiluminescence exposure. Molecular weights (left) are in kDa. \* - These sera (+) are from animals immunized with S-ECP FIA, and are included for comparison.

Figure 5B.





unsuccessful in increasing the IgG titer, but did result in the detection of some proteins by western blot, implying that the treatments enhanced immunogenicity. The alterations in response caused by heat treatment may have been due to increased availability of linear epitopes on ECP proteins following heat denaturation, which could then be recognized on the denatured proteins on the western blot. The reason for the increased level of recognition of certain untreated ECP proteins by sera from mice immunized with PMSF-treated S-ECP is not known. Sulfonylation of the active site serine residue during protease inactivation may have rendered a segment of those proteins more immunogenic, implying that the newly recognized bands may be proenzymes. The molecular weight of the newly recognized band at approximately 33 kDa coincides with the molecular weight of a protease whose activity can be selectively upregulated in the presence of host tissues (MacIntyre et al., 2003); however, it is not known if these are the same protein.

Given the complexity of *P. marinus* ECP, it was not felt that structural factors normally associated with poor immunogenicity, such as glycosylation or high protein solubility, were adequate to explain the globally poor immune response. The diminution in anti-hemolymph IgG titer caused by co-emulsification with S-ECP appears to have confirmed this by demonstrating that S-ECP is capable of actively suppressing the immune response (Fig. 2A). The disparity in mean titer continues through the eighth week (Fig. 2B), which may indicate continued immunosuppression or an initial disruption in the immune response at the time of primary immunization. The former is supported by the continued effect of S-ECP administration on anti-hemolymph primary response ten weeks after initial immunization. The latter is supported by the similarity in the anti-hemolymph titer following the week ten hemolymph boost, despite the presence or absence of S-ECP in the initial immunization.

The similarity in the titers following the week 10 hemolymph boost between mice initially immunized with hemolymph in the presence or absence of S-ECP is partially a function of the apparent difference in the number of recognized epitopes (Fig. 3A). This

is evidenced by a lower maximum reaction rate plateau, indicating that fewer hemolymph epitopes are recognized by those mice that initially received admixed S-ECP. A simple explanation for this effect could be the destruction of epitopes by proteases or other components within the S-ECP. This possibility is rendered less likely by the similar diminishment in maximum reaction rate seen in mice administered hemolymph ten weeks after S-ECP when compared with either naïve mice or mice exposed previously to hemolymph in the absence of S-ECP (Fig. 3A). This leaves the more intriguing possibility that the S-ECP, via its general immunosuppressive activity, caused a reduction in the expressed antibody repertoire. Such reductions in repertoire diversity have been observed in mice in association with depressed T-cell activity (Doenhoff et al., 1979; Dekruyff et al., 1980).

In summary, there appear to be two separate mechanisms for the ECP-mediated immune suppression. The first is the presence of an immunomodulatory surface-active agent, PF68, present in the defined lipid concentrate and co-concentrated with proteins during preparation of S-ECP from the culture supernatant. The second is immunosuppression by constituents of *P. marinus* secreted products.

PF68 is used in suspension culture of animal and insect cells because of its ability to reduce cell death due to shear, and increase recombinant protein production by interaction with the phospholipid membrane (Palomares et al., 2000). PF68 is present in the chemically defined lipid component (#11905, Gibco, Grand Island, NY) of the ODRP-3 medium at a concentration of 100g/L and, thus, in the final media at 1g/L. The 8.4kDa PF68 copolymer molecule is co-concentrated with the ECP proteins during ultrafiltration processing of the culture supernatant, despite the 10kDa molecular weight cutoff of the filtration membrane. Quantification of PF68 demonstrated a 5.8-fold concentration of PF68 with a tenfold volumetric concentration (Table I). This could potentially be due to association of the PF68 with proteins in solution, to steric inability

of the PF68 molecule to pass through a membrane pore, or to micellar structures created by exceeding the critical micellar concentration.

PF68 has been associated with numerous immunomodulatory effects. It inhibits neutrophil random migration, chemotaxis, and adhesion (Lane and Lamkin, 1986; Lane and Krukonis, 1988). Micelles produced by PF68 can cause neutrophil activation and initiate superoxide production, and can render those cells refractory to further activation (Ingram et al., 1992). PF68, when administered on the same day as immunization with sheep erythrocytes, enhances the Arthus reaction and suppresses the delayed-type hypersensitivity reaction (Triolo et al., 1989). PF68 causes hemolysis *in vitro*, but only in concentrations greater than 40 mg/mL (Lowe et al., 1995). *In vitro* exposure of human monocytes to PF68 caused a 3.4-fold increase in procoagulant activity (Janco et al., 1985). PF68 has also been shown to inhibit platelet aggregation in whole human blood (Edwards et al., 1997). It is unknown whether the procoagulant and/or platelet effects of PF68 are related to the hemorrhages observed in mice immunized with S-ECP in CFA. The dose of PF68 administered to mice during immunization with 50 $\mu$ g S-ECP is calculated to be approximately 0.08 g/kg (Table I). This is far lower than the intravenous or intraperitoneal LD<sub>50</sub> in mice of 5.5 g/kg and >10 g/kg, respectively (Pluronic MSDS). It is therefore unlikely that the toxicity seen during S-ECP immunization can be attributed to direct toxic effects of PF68. This does not, however, preclude the possibility that PF68 may interact with constituents of CFA, FIA, or ECP to increase their acute or chronic toxicity.

The pluronic series of polyoxyethylene/polyoxypropylene block copolymers, such as PF68, have a wide range of effects on the interaction between aqueous and lipid phases. Some have been shown to have adjuvant effects, with the degree of adjuvancy dependent on several factors, including molecular weight and hydrophile lipophile balance (HLB) (Hunter et al., 1981; Snippe et al., 1981; Hunter and Bennett, 1984; Hunter et al., 1991). Hunter et al. (Hunter et al., 1981) found that block copolymers with

the best adjuvant properties are those with a HLB below two. No pluronic copolymers tested with HLBs greater than two have adjuvant properties (Hunter et al., 1981). The adjuvant properties of low-HLB pluronics appear to be due to their ability to retain, orient, and present antigens to the immune system on the surface of oil droplets present in the emulsion, with a depot of antigen remaining within the oil which is capable of surface replenishment (Hunter et al., 1981). This contrasts with water-in-oil emulsions such as CFA and FIA, where antigens are present in an aqueous phase contained within oil droplets (Hunter et al., 1981). Pluronic with HLBs greater than two not only fail to retain antigen within the oil phase of the emulsion, but can actually reduce the amount of antigen in that phase below what would have been present in the absence of surfactant (Hunter et al., 1981). Emulsions containing high HLB pluronics are unable to prolong the retention of antigen after injection into mouse footpads, indicating failure to form an antigen depot, and those animals produced little to no specific antibody (Hunter et al., 1981).

PF68 has an extremely high HLB of 29 and its presence in the S-ECP preparation may partially destabilize the Freund's adjuvant emulsion. This destabilization may limit the retention of antigen at the immunization depot, and could thus account for the low titers and predominant IgM response. In combination with the other deleterious immune effects previously described, it is likely that the presence of PF68 is partially responsible for the poor immune response in the mouse. Mouse anti-hemolymph titers are decreased when the hemolymph is administered in the presence of PF68, either as a constituent of the defined lipid supplement, or when it is added to the lipid-free medium (Fig. 4). When PF68 is included in the immunogen, its suppressive effect seems to be somewhat diminished by the presence of lipids in the media prior to concentration. Admixture of lipid-free medium to hemolymph is associated with a diminished response compared to control hemolymph or hemolymph with BSA, though only at the week 8 time point. Surprisingly, the addition of another high HLB non-ionic surfactant, Tween-80

(HLB=15), to the lipid-free medium was not associated with the same decreases in the mean titer seen with PF68. It is possible that its presence did not affect the Freund's emulsion at the concentration present in the media preparation. Alternatively, the 1310 Da Tween-80 molecules may not have concentrated with the proteins during ultrafiltration.

The most striking immunosuppression was caused by ECP depleted of PF68 by DEAE ion exchange chromatography (D-ECP) (Figs. 4A, 4B). The cause of this suppression is not understood, but is currently under investigation. Determination of the mechanism of suppression of the immune response in mice may have implications in the study of the mechanisms of oyster disease caused by *P. marinus*. Oyster defense is composed of innate immune mechanisms such as phagocytosis, encapsulation, and production of reactive oxygen species and lysosomal enzymes. If it can be determined that the deleterious immune effects of ECP in the mice are due to suppression of similar innate mechanisms which then impact the humoral adaptive immune response (e.g. inhibition of phagocytosis by macrophages with failure of antigen presentation), the mouse model may serve as an effective tool in the screening of fractionated ECP for molecules with immune/defense effects. Furthermore, characterization of molecules with such a marked effect on humoral immunity may be valuable in the understanding of other parasitic protozoan diseases.

While the suppression of the humoral immune response caused by PF68 was not nearly as great as that caused by D-ECP, the compounding of these suppressive factors, coupled with the inability to use CFA due to toxicity, was responsible for the failure of previous attempts to produce polyclonal antiserum with a high antigen specific IgG titer, and a panel of monoclonal antibodies. Immunization of mice with D-ECP in CFA followed by boosts in FIA has been associated with increased anti-ECP IgG titers and with a dramatic increase in the number of ECP components detected by western blot (Fig. 5B).

This research demonstrates the potential problems associated with the production of an immunogen in the presence of a common media component. PF68 has an immediate effect on cells, and can affect changes not only in the fluidity and resistance of the plasma membrane, but in cellular metabolism and protein expression (Palomares et al., 2000). PF68 also remains associated with the cell membrane even after removal from PF68-containing media (Palomares et al., 2000). Its presence has the potential to cause artifactual findings in assays of immune cell function, particularly those involved in innate immunity, such as macrophages, neutrophils, and invertebrate hemocytes. The removal of PF68 may be critical for the production of antibodies toward antigenic mixtures, especially those with intrinsic immunosuppressive factors.

## Chapter 5

### Potential novel epitopes in the extracellular products of oyster-product-supplemented *Perkinsus marinus* cells are not detected by subtractive immunization

#### Abstract

The extracellular products (ECP) of the oyster parasite *Perkinsus marinus* may contain virulence factors, including serine proteases. Supplementation of *P. marinus* growth medium with oyster products has been shown to alter the infectivity of the parasite and cause subtle changes in the pattern of ECP produced. A subtractive immunization technique was used in an attempt to create monoclonal antibodies to proteins unique to the ECP produced following parasite exposure to oyster products. While control mice maintained the ability to respond to an unrelated antigen, no detectable serum titer against novel ECP epitopes was detected in the experimental mice. Two separate attempts to produce discriminatory hybridomas also failed to produce any clones secreting ECP-specific antibody.

#### Introduction

The protozoan oyster parasite *Perkinsus marinus* secretes a complex set of extracellular products (ECP) during *in vitro* culture. These products have been demonstrated to adversely affect hemocyte function, including random and directed migration (Garreis et al., 1996) and production of reactive oxygen intermediates (Anderson, 1999) as well as decrease the titer of agglutinins and lysozyme in oyster plasma (Garreis et al., 1996). Feeding oysters *P. marinus* ECP incorporated into liposomes increases the intensity of experimental infections (LaPeyre et al., 1996).

Serine proteases contained within the ECP have been suggested as a possible virulence factor (LaPeyre and Faisal, 1995; LaPeyre et al., 1995; Tall et al., 1999), and have been shown to degrade oyster plasma proteins (Oliver et al., 1999).

In an attempt to create an *in vitro* culture medium which more closely duplicated the *in vivo* environment, oyster products including plasma, whole oyster homogenate, and homogenates of selected organs and tissues have been examined for use as culture supplements. Supplementation with whole oyster homogenates alters *P. marinus* cell size, differentiation, reproductive mechanism, and the constituents of the *P. marinus* ECP, in particular the pattern of secreted serine proteases (See this dissertation, Chapter 3; MacIntyre et al., 2003; Earnhart et al., 2004). Furthermore, supplementation is associated with increased infectivity in experimental infections (Earnhart et al., 2004).

While the modulation of infectivity and pattern of ECP serine protease secretion do not correlate, it is not known if there are other secreted products which are upregulated in response to medium supplementation, and which might be associated with increased infectivity. Due to the complexity of the ECP secreted by *P. marinus*, and the preponderant similarity between the products secreted in the presence or absence of supplementation, a more sensitive method was needed to study subtle differences in the content of the ECP produced by supplemented cells.

Modulation of the murine humoral immune response with the DNA alkylating agent cyclophosphamide (Dong et al., 1995; Bauer and Povirk, 1997) has been used to enhance the ability to distinguish between similar molecules, to increase the likelihood of an immune response against non-immunodominant epitopes, and to bias the humoral response toward differences between two complex sets of antigens (Matthew and Sandrock, 1987; Pukhalsky et al., 1990; Williams et al., 1992; Brooks et al., 1993; Sleister and Rao, 2001; Sleister and Rao, 2002). Mechanistically, cyclophosphamide kills all replicating cells, including any B-lymphocytes induced to proliferate by specific antigen recognition. This deletion of reactive lymphocytes effectively tolerizes the



animal to that antigen set. The subsequent response to an antigen set containing minor protein or epitopic differences is then skewed toward recognition of the novel epitopes. This method could allow the detection of subtle differences between *P. marinus* ECP from cells grown in the presence or absence of oyster supplements and allow further investigation into the significance of these upregulated constituents.

## Materials and Methods

### *Animals*

Six-week-old female BALB/cJ mice from the VIMS breeding colony were used in all experiments. All mice were cared for in accordance with the 1996 National Research Council Guidelines for the Care and Use of Laboratory Animals. All immunizations were administered in divided intraperitoneal and subcutaneous depots and employed a 1:1 emulsion of antigen with Freund's incomplete adjuvant (FIA).

### *Perkinsus marinus* cultures and extracellular product purification

*Perkinsus marinus* isolate P-1 (LaPeyre et al., 1993) was seeded at  $10^6$  cells per mL in 50 mL of ODRP-3 (LaPeyre and Faisal, 1997) medium in 75 cm<sup>2</sup> tissue culture flasks and grown at 27°C under 5% CO<sub>2</sub> for six weeks. The cultures were centrifuged, then filtered to 0.22 µm to remove any residual cells and debris. The ECP was concentrated and purified of media components by ion exchange chromatography as previously described (Earnhart and Kaattari, 2003), and dialyzed against several changes of phosphate buffered saline (PBS; 100mM phosphate, 150mM NaCl, pH 7.4).

For production of ECP from homogenate-pulsed *P. marinus*, cells were grown for six weeks in ODRP-3 medium supplemented with 0.3 mg/mL whole oyster (*C. virginica*, Taylor Resources, Inc., Shelton, WA) homogenate (Earnhart et al., 2004), then extensively washed with, and re-cultured in unsupplemented ODRP-3 medium for six weeks. The ECP was purified as described above from the latter culture supernatants,

and designated pulsed ECP (pECP). Differences between the two ECPs were assessed by gelatin-substrate zymography (Earnhart et al., 2004) and silver stained SDS-PAGE.

### *Subtractive Immunization*

Six mice (A, B, C, D, K, L) were immunized with 50  $\mu$ g each of ECP in FIA in divided intraperitoneal and subcutaneous depots (Table 1). Two control mice (E, F) were administered a similar volume of PBS in FIA. Four of the ECP-immunized mice (C, D, K, L) and the PBS-control mice (E, F) were administered cyclophosphamide 100 mg/kg in PBS intraperitoneally on days 1, 3, and 7 following immunization. Two ECP-immunized mice (A, B) were administered cyclophosphamide 100 mg/kg in PBS on days 3 and 7 only. On day ten, mice A, B, C, D, E, and F were immunized with 50  $\mu$ g pECP in FIA in divided intraperitoneal and subcutaneous depots. To assess the response of non-subtracted mice to the antigens, naïve mice were similarly immunized with 50  $\mu$ g of ECP (G, H) or 50  $\mu$ g pECP (I, J). To demonstrate the preservation of immunocompetence following subtraction, two subtracted mice (K, L) and two naïve mice (M, N) were administered 50  $\mu$ g of bovine serum albumin (BSA) in FIA.

Thirty days after the last immunization, serum from all mice was titrated by ELISA. The anti-ECP and anti-pECP IgG titers were assessed in mice A-J. High protein binding ELISA plates (Costar 3590, Corning, Inc., Corning, NY) were coated with 100 ng of ECP or pECP per well in 100  $\mu$ L of carbonate buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 17.4mM NaHCO<sub>3</sub>, pH 9.7), and residual unbound sites were blocked with 240  $\mu$ L/well of 1% BSA in Tris buffered saline with Tween-20 (TTBS; 50mM Tris, 150mM NaCl, 1mM EDTA, 0.1% Tween-20, pH 7.5). Serial fivefold dilutions of mouse serum in TTBS were applied in duplicate wells and incubated for 1 hour at room temperature. Detection of bound IgG was accomplished with a 1:1000 dilution of an Fc-specific goat-anti-mouse IgG (#115-035-008, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and quantified by the colorimetric detection of the rate of conversion of 2,2'-azinobis(3-

Table 1. Subtractive immunization mouse designation and immunization schedule.

| Mouse | Primary        | Cyclophosphamide        | Secondary       |
|-------|----------------|-------------------------|-----------------|
| A, B  | 50 $\mu$ g ECP | 100 mg/kg, days 3, 7    | 50 $\mu$ g pECP |
| C, D  | 50 $\mu$ g ECP | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g pECP |
| E, F  | PBS            | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g pECP |
| G, H  | —              | —                       | 50 $\mu$ g ECP  |
| I, J  | —              | —                       | 50 $\mu$ g pECP |
| K, L  | 50 $\mu$ g ECP | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g BSA  |
| M, N  | —              | —                       | 50 $\mu$ g BSA  |

ethylbenzthiazoline-sulfonic acid) (ABTS). The anti-BSA titer of all mice was measured similarly, with BSA coated at 100 ng/well, and the unbound sites blocked with TTBS.

#### *Hybridoma production*

On day 32, mouse C was sacrificed for hybridoma production. On day 49, mouse D was boosted with 30 µg pECP intraperitoneally in FIA and 10 µg pECP intravenously, and sacrificed for hybridoma production on day 52. For each fusion, the spleen was removed and the splenocytes were fused with the SP2/0 fusion partner at approximately a 10:1 ratio using 50% polyethylene glycol, resuspended in 10 mL of DMEM, and added to 136 mL of supplemented DMEM (Anonymous, 1986) with 20% FCS and 1% OPI plus 50 mL of SP2/0 conditioned medium and 4 mL of 50X azaserine/hypoxanthine (A9666, Sigma, Inc.). The fused cells were aliquoted at 200 µL per well into ten 96-well plates.

After 12 days, each culture supernatant was screened for anti-pECP activity by ELISA. ELISA wells were coated for 1 hour with 200 ng of pECP protein in carbonate buffer, and blocked with 240 µL/well of 1% BSA in TTBS. Ninety µL of a 1:10 culture supernatant dilution was transferred to the ELISA plates, incubated for one hour, washed, and the bound IgG detected with a 1:1000 dilution of peroxidase-conjugated, goat-anti-mouse IgG antibody, and quantified by colorimetric detection of the conversion of ABTS.

#### **Results**

The silver stained SDS-PAGE demonstrated the expected minor differences between the ECP and the pECP, with several band differences between the two preparations (Fig 1). Gelatin zymography demonstrated upregulated low molecular weight proteases in the pECP. These proteases were in low concentration relative to the high molecular weight forms, and extended incubation was necessary for their clear visualization (Fig. 2).

Mice administered cyclophosphamide on days 1, 3 and 7 maintained tolerance to ECP throughout the course of the experiment (mice C, D) (Fig. 3). Those tolerized only on days 3 and 7 failed to maintain adequate tolerance to ECP (mice A, B). Mice which received the cyclophosphamide regimen in the absence of tolerogen (mice E, F) were able to respond appropriately to a subsequent administration of pECP. Furthermore, mice which were tolerized to ECP were still competent to respond to the unrelated antigen, BSA (mice K-N) (Fig. 4). There was, as expected, considerable cross-reactivity of sera from mice administered ECP versus pECP (mice G-J).

There was no obvious difference between anti-ECP and anti-pECP titers in the subtracted mice; however, since the differences between the two ECPs were expected to be minimal, splenic lymphocytes from the two tolerized mice (mice C, D) were fused to attempt production of discriminatory hybridomas. Despite an excellent rate of hybridoma production of 1 to 2 per well, none secreted antibody specific for any pECP component, as measured by ELISA.

## Discussion

Despite the technically successful subtraction of a complex tolerizing antigen set with maintenance of the ability to respond to a novel antigen, the subtractive immunization failed to resolve any novel antigens, either by screening of serum or fused splenocytes. There are several possible reasons for this failure. First, there may not be any difference between the ECPs at the epitope level. The apparent differences in the ECP may represent differences in processing of a protein common to both ECPs, or may be due to differences in the rate or mechanism of degradation of ECP in the culture medium. Second, it is possible that the changes may be in proteins which are present in subimmunogenic concentrations. This is a particular possibility in the case of the serine proteases, which are present in extremely low concentration despite the high level of activity detected by gelatin zymography. Previous efforts at affinity purification of the

Figure 1. Silver stained SDS-PAGE (10% acrylamide resolving, 4% acrylamide stacking, under non-reducing or reducing conditions) of 5  $\mu$ g of *P. marinus* extracellular products from cells previously grown in unsupplemented medium (ECP) or medium supplemented with whole oyster homogenate (pECP).

Figure 1.

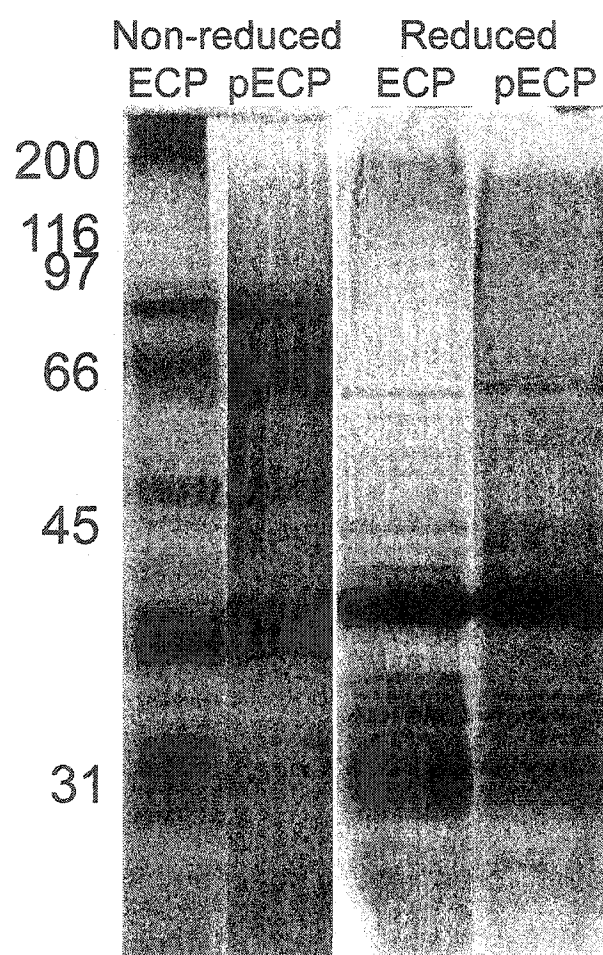


Figure 2. Gelatin substrate SDS-PAGE zymogram (0.2% gelatin, 10% acrylamide resolving, 4% acrylamide stacking, under non-reducing conditions) of *P. marinus* extracellular products from cells previously grown in unsupplemented medium (ECP) or medium supplemented with whole oyster homogenate (pECP). Gels were washed three times for ten minutes with 2.5% Triton-X-100 to renature the proteins, then incubated for two or sixteen hours at 37°C in 0.1 M Tris pH 8.0. Gels were stained in 0.1% Coomassie Brilliant Blue G-250.



Figure 2.

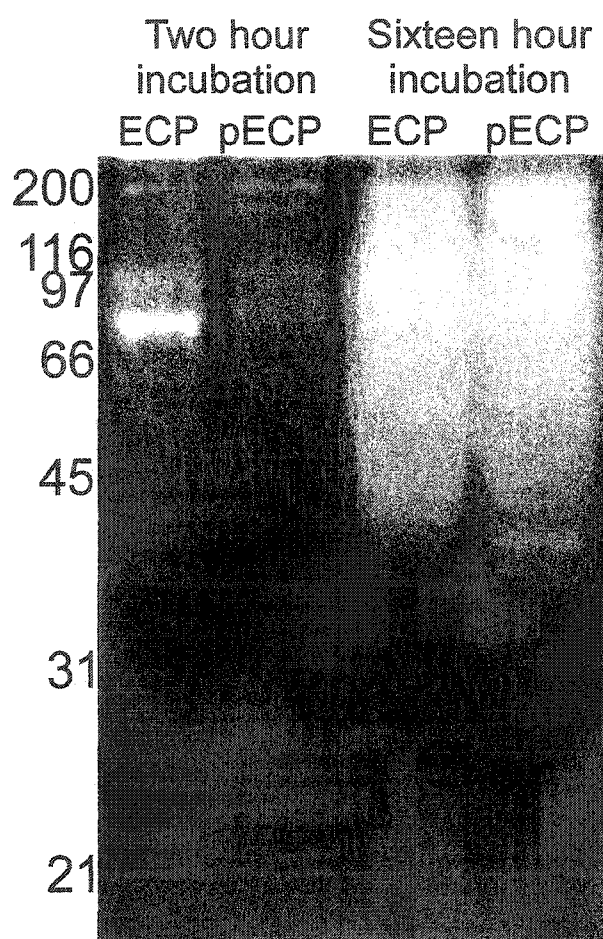


Figure 3.

Subtractive immunization mouse designation and immunization schedule (a), and ELISA titration of IgG-specific anti-ECP (b), or anti-pECP (c) response. ELISA wells were coated with 100 ng of antigen and blocked with 1% BSA in TTBS. Each serum was serially diluted (1:5) in 1% BSA in TTBS and assayed in duplicate. Secondary antibody was a 1:1000 dilution of peroxidase-labeled goat-anti-mouse IgG, and detection was by the chromogenic conversion of ABTS.

Figure 3.

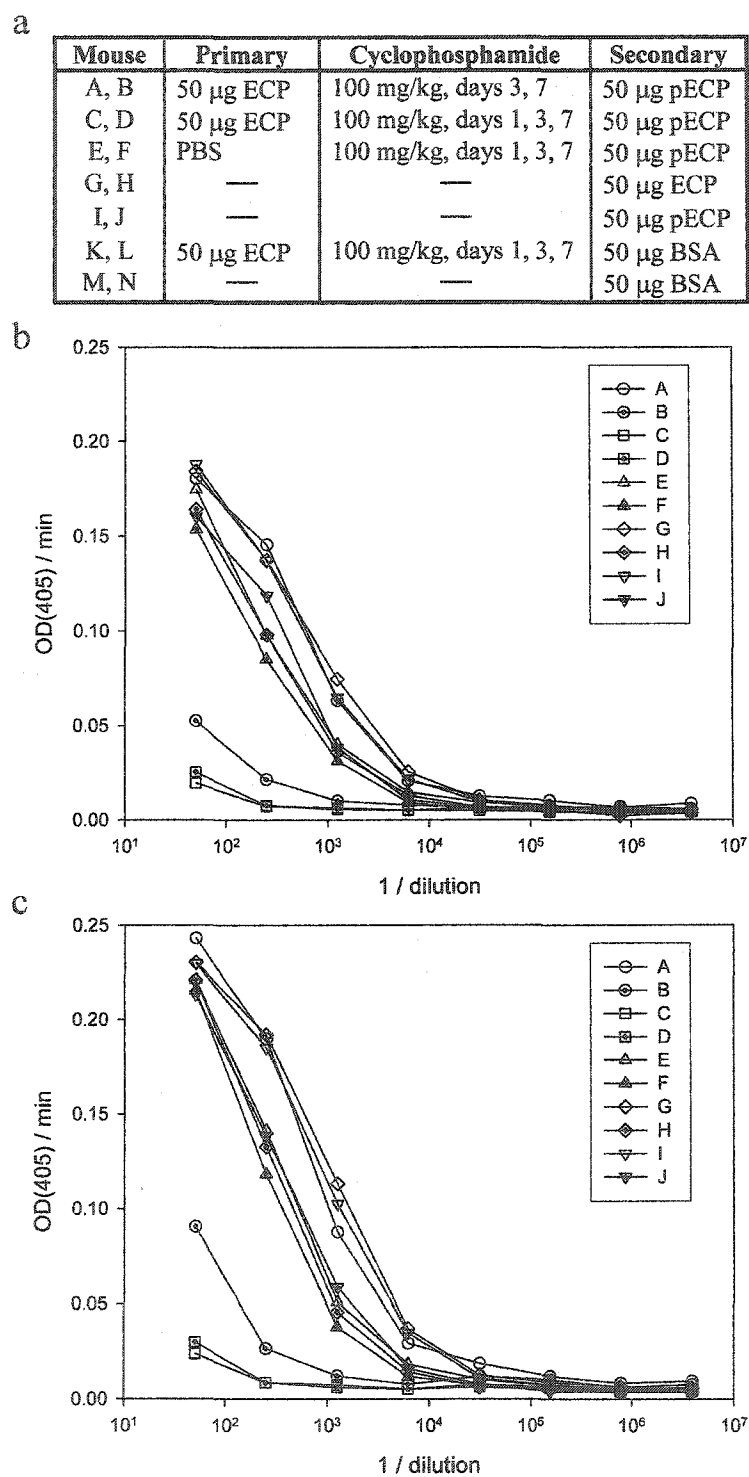


Figure 4.

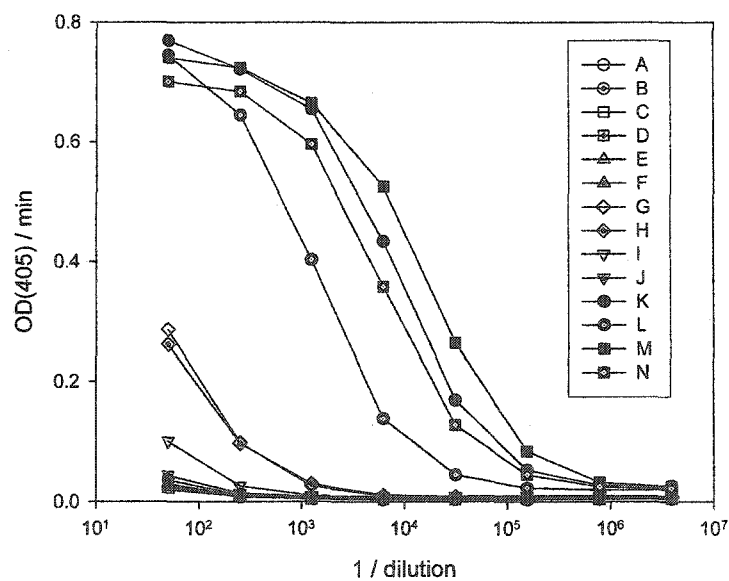
Subtractive immunization mouse designation and immunization schedule (a), and ELISA titration of IgG-specific anti-BSA (b) response. ELISA wells were coated with 100 ng of BSA and blocked with TTBS. Each serum was serially diluted (1:5) in TTBS and assayed in duplicate. Secondary antibody was a 1:1000 dilution of peroxidase-labeled goat-anti-mouse IgG, and detection was by the chromogenic conversion of ABTS.

Figure 4.

a

| Mouse | Primary        | Cyclophosphamide        | Secondary       |
|-------|----------------|-------------------------|-----------------|
| A, B  | 50 $\mu$ g ECP | 100 mg/kg, days 3, 7    | 50 $\mu$ g pECP |
| C, D  | 50 $\mu$ g ECP | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g pECP |
| E, F  | PBS            | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g pECP |
| G, H  | —              | —                       | 50 $\mu$ g ECP  |
| I, J  | —              | —                       | 50 $\mu$ g pECP |
| K, L  | 50 $\mu$ g ECP | 100 mg/kg, days 1, 3, 7 | 50 $\mu$ g BSA  |
| M, N  | —              | —                       | 50 $\mu$ g BSA  |

b



ECP proteases by bacitracin affinity chromatography (van Noort et al., 1991; Faisal et al., 1999) have yielded large increases in activity, but have failed to concentrate the proteases to the point where the protein bands are visible by silver stain, implying that even in concentrated preparations, they are present in sub-nanogram quantities (Earnhart, unpublished results). Alterations in protein expression that occur at this level, while probably biologically significant, may remain undetectable to the murine immune system, even under ideal circumstances. The failure to elicit an immune response could also be due to an intrinsic property of the protein which renders it non-immunogenic, such as the presence of glycosylation moieties which are similar to those found in the mouse.

A third possibility is that the ability of the mouse to respond to particular antigens is diminished by some constituent of the ECP. Previous studies have shown that co-administration of ECP with a highly immunogenic antigen mixture (oyster plasma) severely diminishes the plasma-specific titer (Earnhart and Kaattari, 2003). While the mice did retain the ability to respond to BSA following immunization and subtraction, the second administration of pECP following the subtraction may have limited any ability of the mouse to respond to novel antigen. The mechanism of the diminishment of the humoral response is not known, however, there is evidence in previous studies that *P. marinus* ECP may act as a mitogen (Earnhart, unpublished results). An effort was made, therefore, to delay administration of cyclophosphamide in two of the mice until day three, to allow polyclonal activation and mitogenesis to subside prior to subtraction of antigen specific cells. The failure of these mice to maintain tolerance suggests that if ECP does act as a mitogen, it does not affect the B-lymphocytes. Furthermore, it implies that the antigen specific B-cells have entered a non-replicative stage by day three. Since the ELISA screening assessed IgG secretion, those cells were still able to later undergo class switching, implying the presence of competent T-lymphocyte help. The continued ability of these cells to function adequately does not support the possibility that the failure to

detect novel antigen in the subtractive immunization was due only to ECP-mediated immunosuppression.

Subtractive immunization is a powerful tool to selectively target upregulated antigens in a complex mixture. The failure to distinguish novel antigens provided important information regarding alterations in *P. marinus* ECP constituents by medium supplementation. With regard to the alterations in the protease profile, for example, there are indications of downregulation of some high molecular weight proteases concurrent with the upregulation of the low molecular weight forms. If these proteins are antigenic in the mouse, the subtractive immunization results suggest that the high molecular weight forms may be multi-unit forms of the smaller proteases. This organization may be important in the regulation of protease secretion, stability, or activity. Production of monoclonal antibodies to the shared ECP components may elucidate the relationships and regulation of ECP constituents in standard and supplemented cultures.

## Chapter 6

### Monoclonal antibody analysis of *Perkinsus marinus* extracellular products

#### Abstract

The protozoan oyster parasite *Perkinsus marinus* releases a complex set of extracellular products (ECP) during *in vitro* culture. These products have been previously implicated in parasite virulence, and their expression can be altered by medium supplementation with oyster homogenate. Little is known regarding ECP function, regulation, or mechanism of storage and release. *Perkinsus marinus* ECP were purified from a protein-free medium and used to produce a panel of five monoclonal antibodies. Several of the antibodies recognized series of proteins implying that the ECP may originate from comparatively few parental molecules. The ECP are secreted by several pathways, including the release of one product from the cell wall, and two other products from two morphologically distinct intracellular compartments. Antibodies against separate epitopes on one protein provided information about possible protein structure. A sandwich ELISA format allowed sensitive quantification of that protein and showed significantly reduced protein expression in homogenate supplemented cultures. Immunoaffinity purification has allowed tandem mass spectroscopic amino acid sequencing of that protein. Another antibody was used to characterize the *Perkinsus* cell wall. This antibody specifically bound to trophozoite and tomtom walls, and was used to investigate the morphological and antigenic changes in these walls during thioglycollate-induced formation of prezoosporangia. It was also used to confirm that homogenate supplementation can induce formation of prezoosporangia. This antibody labeled *P. marinus* cells in fixed oyster tissue in a species-specific manner.



## Introduction

*Perkinsus marinus* is a protozoan parasite of the eastern oyster, *Crassostrea virginica*, and has been partly responsible for the decline in oyster populations along the east coast of the United States (Bureson and Ragone-Calvo, 1996; Ford, 1996).

*Perkinsus marinus* is an alveolate protozoan and has been demonstrated by phylogenetic analyses to be basal to the apicomplexans and the dinoflagellates, though it appears to be more closely related to the latter (Fong et al., 1993; Goggin and Barker, 1993; Siddall et al., 1995; Reece et al., 1997; Siddall et al., 1997). The *in vivo* life cycle of *P. marinus* begins with a small, immature trophozoite that enlarges over time into a “signet ring” form, so named for its large vacuole and offset nucleus. This mature trophozoite may then undergo binary fission, or may differentiate into a tomont structure, in which 4 to 64 or more immature trophozoites are formed by palintomic fission within the parental cell wall (Perkins, 1996). *Perkinsus marinus* can also form motile biflagellate zoospores, again by palintomic fission within a differentiated parental trophozoite, the zoosporangium. Zoospores exit the zoosporangium through a discharge tube and pore structure (Perkins, 1996).

During *in vitro* culture in a protein-free, chemically defined medium (ODRP-3; LaPeyre and Faisal, 1997), cellular proliferation is apparently solely by binary fission, and no zoosporulation is seen (Earnhart et al., 2004). Trophozoites can be induced to form prezoosporangia by incubation in fluid thioglycollate medium for 7 to 10 days (Ray, 1952; Ray, 1966; Choi et al., 1989; Bushek et al., 1994; Fisher and Oliver, 1996). Subsequent transfer to sea water has been used to induce further differentiation into zoosporangia with zoospore production (Perkins and Menzel, 1966). Currently, however, and for unknown reasons, prezoosporangia (hypnospores) appear unable to form mature zoospores *in vitro* (Kleinschuster et al., 1994; Perkins, 1996). Supplementation of ODRP-3 medium with oyster tissue homogenates may induce a partial transition to the

prezoosporangium stage without thioglycollate incubation, as evidenced by the appearance of enlarged cells with thickened cell walls (Earnhart et al., 2004).

The mechanisms by which *P. marinus* causes disease are not clear, though there is evidence that extracellular products (ECP) may play a role in virulence. *Perkinsus marinus* ECP have been shown to blunt the defensive generation of reactive oxygen intermediates by oyster hemocytes (Volety and Chu, 1995; Anderson, 1999). ECP and, in particular, the serine protease components, decrease random and chemotactic oyster hemocyte migration (Garreis et al., 1996), lysozyme activity, and plasma agglutinin titer (Garreis et al., 1996), and increase experimental infection level after oral administration of liposome-encapsulated ECP (LaPeyre et al., 1996). Oyster plasma proteins are also susceptible to cleavage by these proteases (Oliver et al., 1999). The secretion pattern of the serine proteases can be modulated by supplementation of ODRP-3 medium with homogenized oyster tissues (See this dissertation, Chapter 3; MacIntyre et al., 2003; Earnhart et al., 2004), though this modulation has not been definitively linked to changes in parasite virulence. *Perkinsus marinus* ECP are highly immunosuppressive in mice, indicating that there may be an effect on some basic component of innate immunity (Earnhart and Kaattari, 2003).

Assessment of induction, expression levels, mechanisms of elaboration, and functions of possible *P. marinus* ECP virulence factors in ECP requires sensitive and specific methods of detection and purification. Attempts have been made to produce antibodies, mostly as polyclonal antisera, using whole cell immunogens (Dungan and Roberson, 1993; Romestand et al., 2001), lysed cells (Choi et al., 1991; Montes et al., 2002), or extracellular products (Ottinger et al., 2001). Although these antibodies have been successfully used to label *P. marinus in vivo* (Dungan and Roberson, 1993), identify a major cell wall component (Montes et al., 2002), and quantify *in vitro* proliferation (Romestand et al., 2001) as well as *in vivo* parasite load (Ottinger et al., 2001), there have been problems with specificity, with antibodies failing to identify some parasite life

stages (Choi et al., 1991) or crossreacting with other microorganisms or with oyster tissues (Dungan and Roberson, 1993; Dungan et al., 2000; Ottinger et al., 2001; Bushek et al., 2002). The goal of the current study was to produce monoclonal antibodies for characterization of one or more *P. marinus* ECP, including quantification, determination of storage and release mechanisms, and purification to allow amino acid sequencing.

## Materials and methods

### *In vitro propagation of Perkinsus marinus and production of ECP*

*Perkinsus marinus* isolate P-1 (LaPeyre et al., 1993) was seeded at  $10^6$  cells per mL in 50 mL of ODRP-3 (LaPeyre and Faisal, 1997) medium in 75 cm<sup>2</sup> tissue culture flasks and grown at 27°C under 5% CO<sub>2</sub> for six weeks. The cultures were centrifuged and filtered to 0.22 µm to remove cells and debris. The ECP were purified and concentrated by ion exchange chromatography as previously described, and dialyzed against several changes of phosphate buffered saline (PBS) (Earnhart and Kaattari, 2003).

### *Immunization and hybridoma production*

Six-week-old female BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were cared for in accordance with the 1996 National Research Council Guidelines for the Care and Use of Laboratory Animals. All immunizations employed a 1:1 emulsion of antigen with either Freund's complete (CFA) or incomplete (FIA) adjuvant. The mouse used for hybridoma production was immunized with 50 µg ECP in CFA, followed by FIA boosts with 20 µg on week 4 and 10 µg on week 8. On week 12, the mouse was given a final 5 µg boost in FIA intraperitoneally and a 5 µg intravenous injection of antigen without adjuvant. Three days later, the spleen was removed and the splenocytes were fused with the SP2/0 fusion partner at approximately an 8:1 ratio using 50% polyethylene glycol, resuspended in 10 mL of DMEM, and added to 136 mL of supplemented DMEM (Anonymous, 1986) with 20% FCS and 1% OPI plus

50 mL of SP2/0 conditioned medium and 4 mL of 50X azaserine/hypoxanthine (A9666, Sigma, Inc.), and aliquoted at 200  $\mu$ L per well into ten 96-well plates.

After 12 days, a 1:10 dilution of each culture supernatant was screened for anti-ECP activity by ELISA. ELISA wells (Costar 3590, Corning, Inc., Corning, NY) were coated for 1 hour with 25 ng of antigen in carbonate buffer, and blocked with 240  $\mu$ L/well of 1% bovine serum albumin (BSA) in Tris buffered saline with Tween-20 (Earnhart and Kaattari, 2003). The culture supernatant dilution was incubated for one hour, washed, and the bound IgG detected with a 1:1000 dilution of peroxidase-conjugated, goat-anti-mouse IgG antibody (#115-035-008, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and quantified by colorimetric detection of the conversion of 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS).

All ELISA positive colonies were rescreened by immunoblot. Twenty  $\mu$ g of ECP were separated on preparative SDS-PAGE gels (Laemmli, 1970), and transferred to PVDF membranes. The blots were blocked with TTBS, and screened with 1:10 dilutions of culture supernatant using a Surfblot apparatus (Earnhart and Kaattari, 2003). Secondary detection was by peroxidase-conjugated, goat-anti-mouse IgG. Reactive bands were visualized by film exposure using enhanced chemiluminescence reagents (Biomax MR-1, Eastman Kodak, Rochester, NY; ECL, Amersham Biosciences, Piscataway, NJ).

Selected hybridomas were cloned two to three times by limiting dilution (Harlow and Lane, 1988) and expanded for production of antibodies by ascites formation in pristane-primed mice (Harlow and Lane, 1988). Antibody was purified from the ascites fluid by protein-A conjugated Sepharose beads (153-6159, Bio-Rad Laboratories) and the IgG subisotype was determined by ELISA (ISO2, Sigma, Inc.). To differentiate between antibody recognition of protein or carbohydrate epitopes, 200 ng ECP was treated with periodate in an ELISA format as previously described (Woodward et al., 1985).

Antibodies were assessed for utility in a sandwich ELISA format using a matrix screening format. Two hundred ng of each antibody were coated onto ELISA plates in carbonate buffer, and the plates were blocked with 1% BSA in TTBS. All wells were then incubated with 500 ng *P. marinus* ECP for 1 hour. All possible combinations of antibodies were tested using NHS-biotin labeled antibodies (Rosenberg, 1996) to detect the bound ECP. Biotinylated antibodies (200 ng/well) were incubated for 1 hour, and detected by 1:1000 dilution of horseradish peroxidase conjugated streptavidin and colorimetric detection using ABTS.

#### *Immunoaffinity purification and de novo amino acid sequencing*

Two mL of protein-A-conjugated Sepharose beads were equilibrated in the manufacturer's binding buffer and added to 6.5 mg of purified antibody in 8 mL of binding buffer. The beads were incubated with end-over-end mixing for 90 minutes, washed twice with 20 mL of binding buffer, resuspended in 10 mL of 20 mM dimethylpimelimidate in binding buffer for 30 minutes, then incubated for two hours with 0.2 M ethanolamine, pH 8. Residual non-covalently bound IgG was removed by washing the beads in 100 mM glycine, pH 3.

For antigen purification, the antibody-coated beads were equilibrated in PBS and packed by gravity into a 5 mL column. Two hundred µg of ECP were treated with 2 mM phenylmethylsulfonyl fluoride to inactivate the endogenous serine proteases, diluted to 10 mL in PBS, and run through the column by gravity. The column was washed with 30 mL of PBS, and the bound antigen eluted with 100 mM glycine, pH 2, collected in 200 µL fractions, and neutralized with 10 µL of 1 M sodium phosphate, pH 8. Fractions containing protein were pooled and concentrated by ultrafiltration (Microcon-10). The purity of the product was assessed by reducing and non-reducing SDS-PAGE.

The antigen purified by antibody 1B3 was subjected to two dimensional electrophoresis (Invitrogen, Carlsbad, CA), with or without pI markers, using pH 3-10

IPG strips, and a 4-16% acrylamide second dimension. One gel was stained using a mass spectroscopy-compatible silver stain (SilverQuest, Invitrogen), and a spot was excised for tandem mass spectroscopy amino acid sequencing at Eastern Virginia Medical School.

*Quantification of p107/p102 in a sandwich ELISA format*

ELISA wells were coated with 200 ng of antibody 5G9 in carbonate buffer, and blocked with 1% BSA in TTBS. *Perkinsus marinus* cultures were grown for four weeks in 75 cm<sup>2</sup> flasks in ODRP-3 medium supplemented with 0.25 mg/mL of tissue homogenate from *Crassostrea ariakensis* oysters or *C. virginica* oysters from Louisiana, Tangier Sound (Chesapeake Bay, Virginia), the CROSBreed program, or Washington state. Control cultures were unsupplemented. The cells from these cultures had been used in a prior study of infectivity (Earnhart et al., 2004). For titration of p107/p102, each culture supernatant sample was compared with a standard ECP. This standard ECP was determined to contain 10 µg/mL of p107/p102 by titration of activity against a known quantity of affinity purified p107/p102 protein in a manner identical to that described below.

Each culture supernatant, negative control unseeded medium, and standard *P. marinus* ECP was titered by serial twofold dilutions in 1% BSA in TTBS in duplicate wells and incubated for one hour. The plates were then washed and incubated with 200 ng of biotinylated 1B3 for 1 hour, followed by a 1 hour incubation with 100 µL of a 1:1000 dilution of horseradish peroxidase-conjugated streptavidin. The rate of peroxidase-catalyzed conversion of ABTS substrate was measured spectrophotometrically. The quantity of p107/p102 was determined by fitting a sigmoidal curve to the rate data from each dilution series using a four-parameter logistic equation, and interpolation of concentration at the value corresponding to 30% of the reaction rate plateau of the standard ECP for that plate (SigmaPlot, SPSS, Inc., Chicago, IL) (Arkoosh and Kaattari, 1990).

To confirm that the oyster homogenate did not interfere with antigen binding, ECP-spiked homogenate samples from three different oyster species were assessed. Whole tissue homogenates from *Crassostrea gigas*, *C. virginica*, and *C. ariakensis* were adjusted to 3.0 mg protein/mL and spiked with an equal volume of standard ECP containing 20 µg/mL of p107/p102. Serial dilutions of each spiked homogenate were made in duplicate using both TTBS and 1.5 mg/mL homogenate as diluents. These dilutions were assayed and p107/p102 quantified using the 5G9/biotinylated 1B3 ELISA described above.

#### *Fluorescence immunostaining*

*Perkinsus marinus* cells grown in ODRP-3 medium were harvested after one and eight weeks of culture and  $10^6$  cells were applied to slides (Superfrost plus, Fisher Scientific) by cytospin. The cells were air dried, blocked with 1% BSA in PBS for one hour, incubated in a 1:1000 dilution of each monoclonal antibody for one hour, washed three times in PBS, incubated in a 1:64 dilution of a fluorescein-conjugated Fc-specific goat-anti-mouse IgG (F8264, Sigma, Inc.) for one hour, and again washed. *Perkinsus marinus* hypnospores (prezoosporangia) were created by incubation of cells in thioglycollate medium with 20% (v/v) chemically defined lipid supplement (#11905, Gibco, Grand Island, NY) (Ray, 1952; Chu and Lund, 2004). After 10 days of incubation, the cells were pelleted by centrifugation and stained with antibody 5A2 as described above. Another  $10^6$  cells were stained with Lugol's iodine solution. The cells in both experiments were photographed using a Nikon DXM1200 digital camera or an Olympus D1000 35mm camera on an Olympus BX51 microscope.

#### *Immunogold electron microscopy*

*Perkinsus marinus* cells from four-week-old unsupplemented cultures, from cultures supplemented with 0.3 mg/mL oyster (*C. virginica* WA) homogenate, from cells

incubated in thioglycollate as described above, or from thioglycollate-incubated cells transferred for one week into artificial sea water were fixed in matched-osmolality 1% glutaraldehyde/4% paraformaldehyde for 1 hour. The fixed cells were washed once in 0.1 M sodium cacodylate, enrobed for 1 hour in 2% agar / 0.1 M sodium cacodylate, and dehydrated through a graded ethanol series in 10% increments, with 15 minutes per step and two changes at the 100% level. The temperature was lowered to and maintained at  $-20^{\circ}\text{C}$  after the 30% step. The dehydrated cells were infiltrated with Lowicryl HM20 for 36 hours, and the blocks were polymerized with long-wave UV light (360 nm, 15 W at 1 meter). Sections were cut at 90 nm on a Reichert-Jung ultramicrotome and mounted on gold grids coated with carbon-stabilized Formvar.

The sections were blocked with 0.5% BSA in PBS for 30 minutes, transferred to a 1:250 dilution of antibody 5A2 in blocking solution, incubated for 1 hour, and washed five times in PBS. Negative controls were included that omitted primary antibody. The sections were then incubated for 1 hour in a 1:250 dilution of 10 nm colloidal gold-conjugated goat-anti-mouse IgG antibody (G-7652, Sigma, Inc.), washed five times in PBS, incubated for 5 minutes in 1% glutaraldehyde to fix the label, washed with ddH<sub>2</sub>O, air dried, stained for 15 minutes with saturated uranyl acetate, and examined on a Zeiss CEM 902 transmission electron microscope.

### *Immunohistochemistry*

Infected and uninfected oysters were fixed in neutral buffered formalin and embedded in paraffin. Six  $\mu\text{m}$  sections were cut, mounted onto slides (Superfrost plus, Fisher Scientific), deparaffinized in xylene, and rehydrated through a graded ethanol series. Sections were blocked in PBS with 3% BSA for one hour, incubated in a 1:500 dilution of each monoclonal antibody for one hour, washed three times in PBS, incubated in a 1:250 dilution of peroxidase-conjugated goat-anti-mouse IgG for one hour, and again washed. Control slides omitted the primary or both antibodies. The bound antibodies



were localized by the peroxidase-mediated conversion of aminoethylcarbazole to a colored product. The sections were counterstained in a 1% solution of Fast Green.

## Results

### *Monoclonal antibody production*

Thirty-eight clones from ELISA positive wells were initially expanded. After further screening and selection, nine hybridomas were cloned. Of those nine, several recognized the same protein(s), and were assessed for their utility in a sandwich-type ELISA format. Five monoclonal antibodies, each with a different epitope specificity, were chosen for further investigation (Fig. 1; Table 1). None of the combinations of antibodies with similar immunoblot reactivity reacted with different epitopes, precluding their use in a sandwich format. However, the immunoblot-negative antibody 1B3 did react with the same protein as antibody 5G9 (Fig. 2).

Several of the antibodies, despite their monospecificity, recognized series of proteins by immunoblot (Fig. 1). Periodate oxidation of carbohydrates did not alter antibody recognition, indicating that the epitopes recognized are likely not carbohydrates (Fig. 3). Rather than elimination of activity, there was an increase in the maximum rate of change in substrate conversion after periodate treatment for both antibodies 1B3 and 5G9, which may represent improved access to the protein epitope following oxidation of the carbohydrates.

### *Immunoaffinity purification and de novo amino acid sequencing*

Immunoaffinity purification of proteins recognized by antibodies 5G9 and 1B3 demonstrated that both recognize a protein doublet of 107 and 102 kDa (p107/p102), which concurred with the immunoblot results for antibody 5G9 (Fig. 1); however, in addition to those shared bands, antibody 1B3 purified an 81 kDa protein (Fig. 4). Under reducing conditions, there is an intense 42 kDa band in both purifications.

Using two dimensional electrophoresis of the 1B3 purification product, it was determined that the immunopurified 42 kDa band was heterogeneous and composed of approximately eight spots encompassing a wide range of isoelectric points (pI) (Fig. 5). Protein spots occurred at a pI of 4.9, and in the range of pI 6.5 to 7.3, with the most prominent spot occurring at a pI of 6.85. The protein also displayed some minor molecular weight variation, with two series of spots occurring in a range of approximately 1 kDa. The most intensely staining spot (Fig. 5) was selected for *de novo* amino acid sequencing by tandem mass spectrometry. The DeNovoX software package provided numerous short amino acid sequences (Table 2); however, BLAST searches for short, nearly exact matches of the amino acid sequences did not yield consistent protein similarities.

#### *Quantification of p107/p102 in a sandwich ELISA format*

Since antibody 1B3 recognized an 81 kDa protein in addition to the antigens recognized by 5G9 (Fig. 4), it was necessary to use the more restrictive 5G9 as the capture antibody in the sandwich ELISA. This eliminated the possibility of blockade of detection of p107/p102 by the 81 kDa protein (Fig.2). There were marked differences in the level of p107 present in whole oyster homogenate-supplemented versus unsupplemented *P. marinus* cultures (Fig. 6a). While the unsupplemented ODRP-3 culture had a concentration similar to that of the standard ECP, all supplemented cultures demonstrated depressed levels of the p107 protein. This effect was less pronounced in the cultures supplemented with homogenate from *C. virginica* oysters from Washington state. The combination of standard ECP with culture supplements did not demonstrate any problem with blocking or epitope destruction (Fig. 6b). In all cases, the measured level of p107 in the spiked sample was similar to or slightly greater than the expected value of 200 ng/mL.

### *Fluorescence immunostaining*

Antibody 1B3 stained apparently internal vesicular structures within the *P. marinus* cells; however, these structures were only seen in the one week old cultures, and then only in a subset of cells (Fig. 7a). The 5G9 antibody, which detects a subset of the proteins recognized by 1B3 did not stain any structure at either the one- or eight-week time point. At both time points, antibody 9E12 primarily stained somewhat round, as well as more diffuse, filamentous, intracellular structures (Fig. 7b, c). Antibody 5A2 brightly stained the cell exterior at both time points (Fig. 7d, e). An apparent internal wall forming during cell division was visible in some cells, indicating that the antigen is present even at the earliest stages of wall formation (Fig. 7f). The 5H11 antibody did not stain any structure at either time point.

*Perkinsus marinus* cells incubated in lipid-supplemented FTM displayed a range of sizes (Fig. 7f, g). The smallest cells were similar in size to unincubated trophozoites. These cells stained brightly with antibody 5A2, and did not stain with Lugol's iodine solution. At the other size extreme were extremely enlarged cells with thickened cell walls having the appearance of prezoosporangia. The walls of these cells stained darkly with Lugol's iodine, but stained very weakly with antibody 5A2. Where staining occurred, it often appeared to be patchy. There were also cells which were of intermediate size, and of intermediate staining intensity. These cells had somewhat thickened cell walls which stained lightly with Lugol's iodine. They displayed more intense staining with antibody 5A2 than did the prezoosporangia, but again the antigen appeared to be distributed in patches, in contrast to the homogeneously bright stain seen in trophozoites. There was no fluorescent staining in any of the control treatments.

### *Immunogold electron microscopy*

Trophozoites from unsupplemented *P. marinus* cultures, as well as trophozoites and tomonts from supplemented cultures, were tested by immunogold labeling for

reactivity with antibody 5A2. Antibody 5A2 strongly labeled the cell wall of trophozoites from both unsupplemented and homogenate-supplemented cultures, as well as tomont structures in the latter (Fig. 8). Trophozoites from unsupplemented cultures typically had a thin layer of somewhat loosely adherent cell wall material which was 5A2-reactive, and which became separated from the cell wall during fixation (Fig. 8a). Trophozoites from supplemented cultures were typically larger, and had a somewhat thicker, heavily labeled cell wall (Fig. 8b).

Cells in supplemented cultures frequently replicated by palintomic fission within the cell wall of a parental trophozoite. Antibody 5A2 labeled both the parental wall as well as the newly formed walls of the internal daughter trophozoites in this tomont structure (Fig. 8c). Enlarged trophozoites resembling prezoosporangia were occasionally found in the supplemented cultures. These cells had a two-layered wall, with a heavily-labeled external wall that appeared fibrillar and loosely attached to a lightly-labeled, more dense internal cell wall (Fig. 8d). Minimal labeling was observed in negative controls, with typically one to two beads per *P. marinus* cell cross-section.

Prezoosporangia created in thioglycollate medium frequently had a two-layered cell wall morphology. Many cells had a thick, dense, lightly-labeled internal wall with an outer, patchy layer of heavily labeled, fibrillar wall material (Fig. 9a). Where this outer wall material was completely removed, only the outermost surface of remaining wall was moderately labeled (Fig. 9b). In some cases, the entire heavily-labeled outer cell wall appeared to have detached from the inner cell wall structure as a sheet (Fig. 9c). Following incubation in artificial sea water to initiate palintomic division into zoosporangia containing prezoospores, loosely-adherent, heavily labeled outer wall fragments remained over a thinner, lightly-labeled inner wall (Fig. 9d) in a pattern identical to that seen in prezoosporangia. In contrast with the cells created during palintomic replication of trophozoites in tomonts (Fig. 8d), the prezoospore walls did not label (Fig. 9d).

Figure 1.

Immunoblot (a) of 30  $\mu$ g of ECP under non-reducing conditions using 1:1000 dilutions of purified monoclonal antibodies. Secondary detection was by a 1:10000 dilution of peroxidase-labeled goat-anti-mouse IgG. Reactivity was detected by enhanced chemiluminescence and film exposure, and total protein was stained with colloidal gold. Molecular weights (right) are in kDa.

Figure 1.

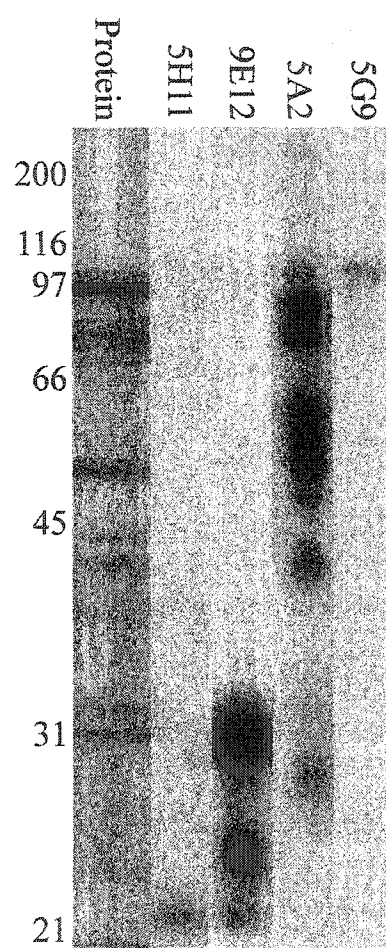


Table 1. Proteins from *P. marinus* ECP recognized by individual monoclonal antibodies.

| Antibody | Sub-isotype      | Proteins recognized (kDa)            |
|----------|------------------|--------------------------------------|
| 1H3      | IgG <sub>1</sub> | 22                                   |
| 9E12     | IgG <sub>1</sub> | 31, 24, 22                           |
| 5A2      | IgG <sub>3</sub> | 235, 107, 90, 74, 61, 54, 50, 41, 28 |
| 5G9      | IgG <sub>1</sub> | 107, 102                             |
| 1B3      | IgG <sub>1</sub> | 107, 102, 81                         |

Figure 2.

Assessment of antibody utility in a sandwich ELISA format. ELISA plates were coated with 200 ng of antibody 5G9 (a) or 1B3 (b), blocked, incubated with 1% BSA in TTBS, and incubated with *P. marinus* ECP (5 µg/mL) for 1 hour. After washing, biotinylated antibodies (200 ng/well) were incubated for 1 hour, and detected by 1:1000 dilution of horseradish peroxidase conjugated streptavidin and colorimetric detection using ABTS.



Figure 2.

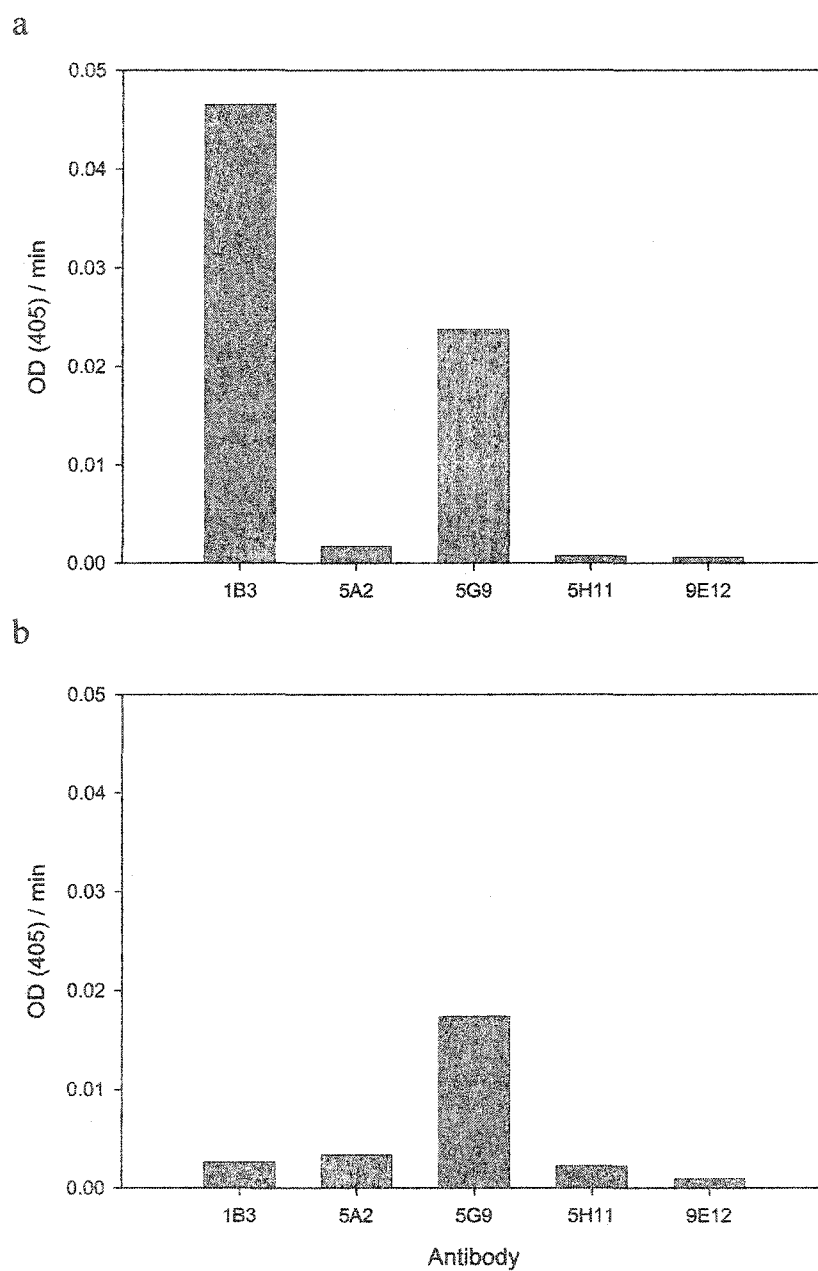


Figure 3.

ELISA of periodate-treated and control ECP. ECP was coated onto plates at 2  $\mu\text{g/mL}$ , and residual sites blocked with TTBS. The treated plate was treated for 1 hour with 5 mM periodate in 50 mM acetate buffer, pH 4.5. Control plate was incubated in acetate buffer. Both plates were then incubated for 30 minutes in 50 mM sodium borohydride in PBS, and washed with TTBS. Serial dilutions of each antibody were made in duplicate, and incubated for 1 hour at room temperature. Secondary detection was with a peroxidase-conjugated goat-anti-mouse IgG antibody.

Figure 3.

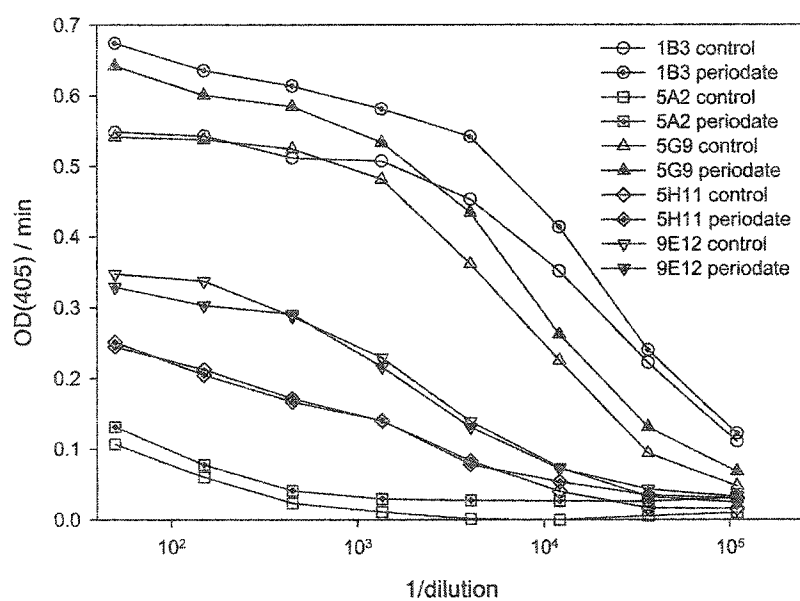


Figure 4.

Silver stained SDS-PAGE (12% resolving, 4% stacking) under non-reducing and reducing ( $\beta$ -mercaptoethanol) conditions of the ECP affinity purification product using antibodies 1B3 and 5G9. Molecular weights are in kDa.

Figure 4.

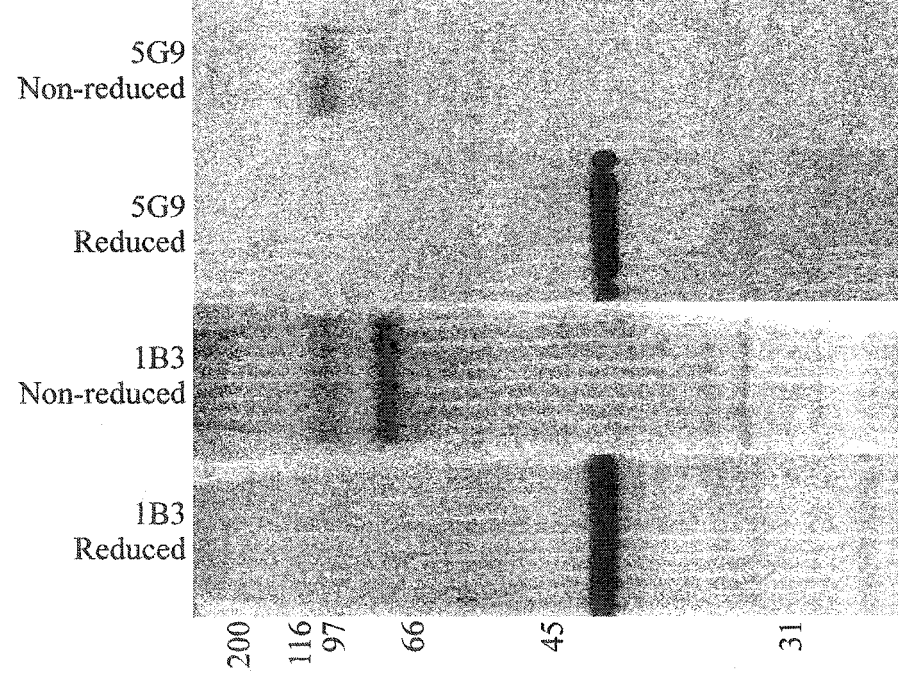


Figure 5.

Two dimensional separation of protein affinity purified by antibody 1B3, using a 3-10 pI range first dimension and a 4-16% gradient tris-tricine polyacrylamide gel second dimension. Isoelectric points were estimated by using pI markers run concurrently with p107/p102 in a separate gel (not shown). Molecular weights (left) are in kDa. Mouse IgG heavy chain (IgG HC) and light chain (IgG LC) are present due to minor leakage of antibody 1B3 from the affinity column during elution. The protein spot selected for *de novo* amino acid sequence analysis by tandem mass spectrometry is designated with an asterisk (\*).

Figure 5.

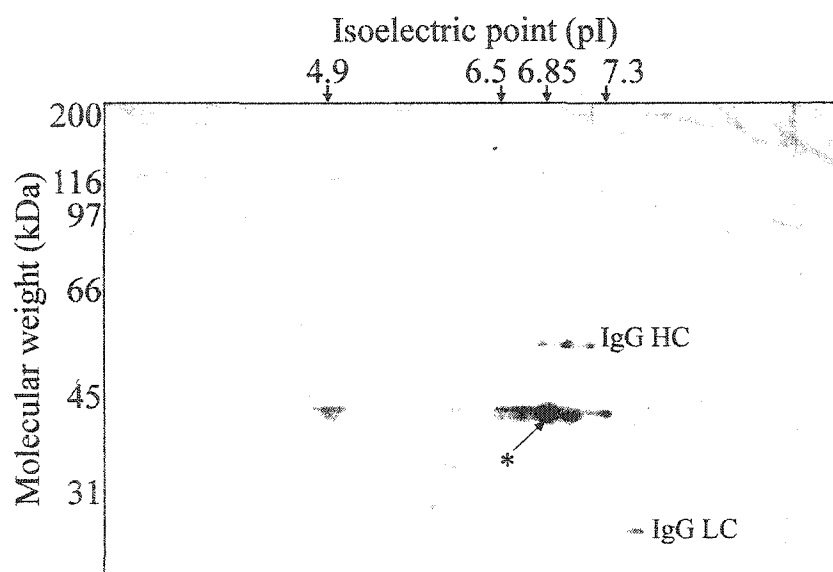


Table 2. Selected p42 tandem mass spectrometry *de novo* amino acid sequences.

| Amino acid sequence | Relative probability |
|---------------------|----------------------|
| TPWASLGLSLHR        | 95.1                 |
| TPLVLSSS            | 71.3                 |
| MLFYEHYQ            | 47.5                 |
| SPTTTEDWYLS         | 42.4                 |
| YYYYYYY             | 28.4                 |
| QTPLVL              | 28.1                 |
| ENAGLK              | 22.8                 |
| LLFGGD              | 22.0                 |
| LAMRMPP             | 19.6                 |



Figure 6.

Quantification of p107/p102 (a) in unsupplemented medium (ODRP-3) or the same medium supplemented with 0.25 mg/mL whole oyster homogenate derived from *Crassostrea ariakensis* (Ca), or from populations of *C. virginica* from Louisiana (CvLA), Tangier sound, Virginia (CvTG), the CROSBreed program (CvXB) or Washington state (CvWA). Quantification of p107/p102 (b) from homogenates of *C. ariakensis* (Ca), *C. gigas* (Cg), or *C. virginica* (CvWA) spiked with 10 µg/mL of p107/p102 in standard ECP. ELISA plates were coated with 200 ng of antibody 5G9 and blocked with 1% BSA in TTBS. Serial twofold dilutions of each sample were made with homogenate (gray bars) or TTBS (hatched bars) and assessed in duplicate. The bound antigen was then detected by incubation with 200 ng/well of biotinylated antibody 1B3 followed by incubation with a 1:1000 dilution of peroxidase-conjugated streptavidin. The titrated rates of conversion of ABTS by each sample were compared with a standard ECP for quantification of p107/p102.

Figure 6.

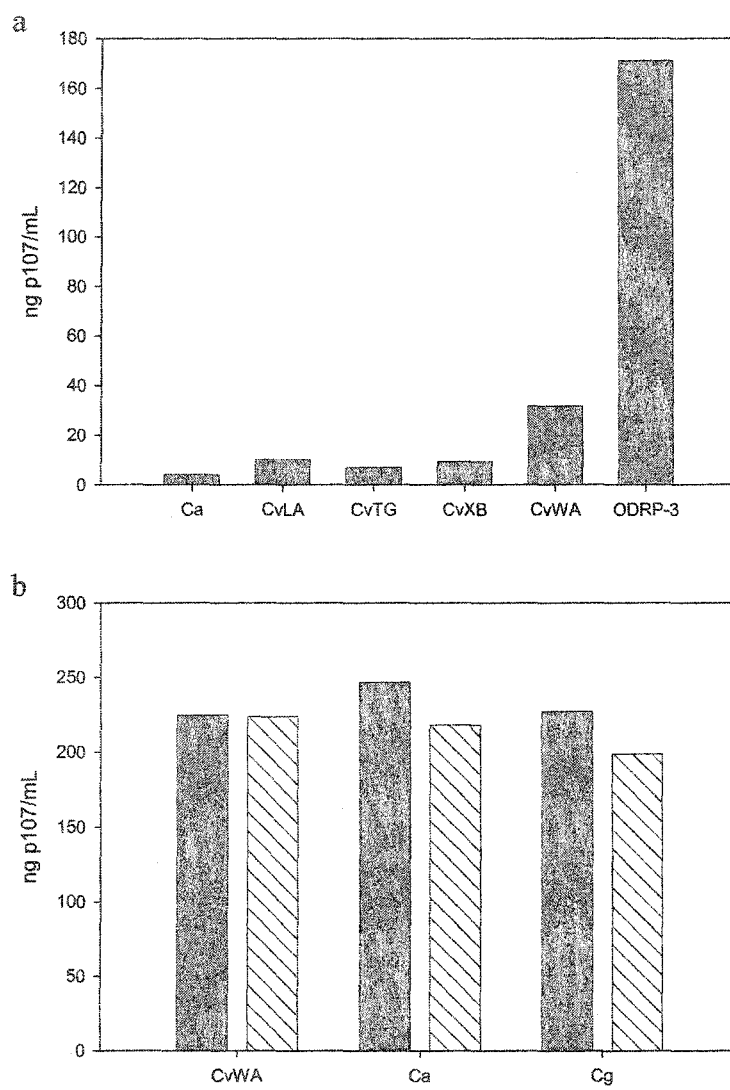


Figure 7.

Immunofluorescent staining of 1 week old (a, b, d) and 8 week old (c, e) *P. marinus* cells grown in ODRP-3 medium, or 8 week old cultures incubated in lipid-supplemented thioglycollate medium for 10 days (f, g). Antibodies 1B3 (a), 9E12 (b, c), and 5A2 (d, e, f) were applied at a 1:1000 dilution to *P. marinus* cells which had been applied by cytopspin to slides, dried, and blocked with 1% BSA in TTBS. Following three washes, the bound monoclonal antibodies were detected with a 1:64 dilution of fluorescein-labeled goat-anti-mouse IgG antibody. Cells which were incubated in thioglycollate were washed with PBS, blocked with 1% BSA in PBS, incubated with a 1:1000 dilution of antibody 5A2, washed, incubated in a 1:200 dilution of fluorescein-labeled goat-anti-mouse IgG, washed, and resuspended in PBS (f). Some thioglycollate incubated cells were washed in PBS, then stained with Lugol's iodine (g). Cells were photographed using a fluorescein filter set (a-f) or phase contrast microscopy (g) at an original magnification of 1000X (a-e) or 400X (f, g). In thioglycollate cultures (f, g), prezoosporangia are marked with an asterisk (\*), trophozoites which are partially enlarged are marked with arrowheads, and non-enlarged trophozoites with arrows.

Figure 7.

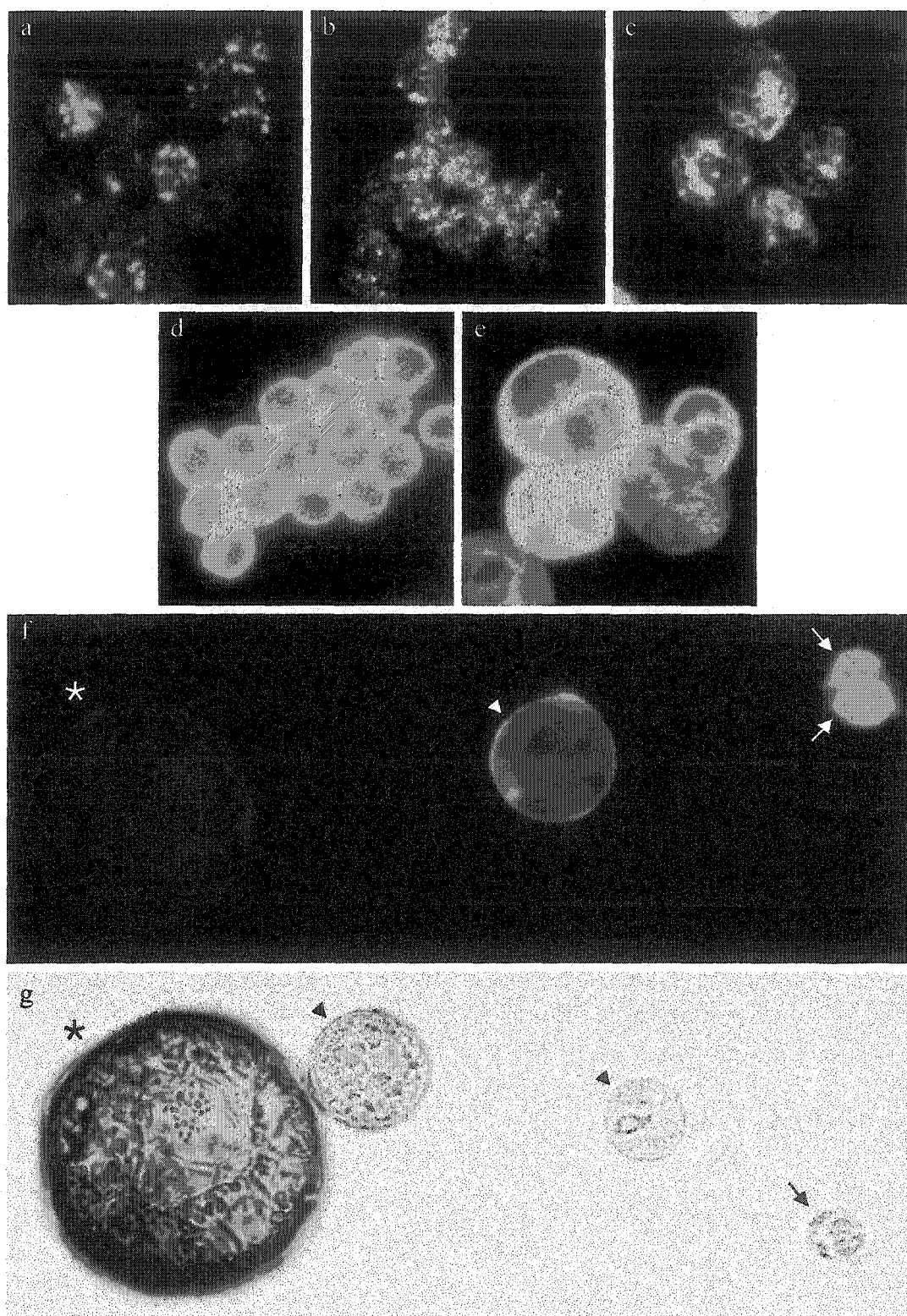


Figure 8.

Immunogold electron microscopy of *P. marinus* trophozoites (a, b), tomont (c), and thick-walled trophozoite (d) which were grown in unsupplemented ODRP-3 medium (a) or the same medium supplemented with 0.3 mg/mL of whole oyster tissue homogenate (b, c, d). Arrows indicate the antibody 5A2 reactive cell wall, which in some cases (a) is separated from the plasma membrane (open arrowhead). Non-5A2-reactive cell wall (d) which resembles a prezoosporangium wall is indicated by a closed arrowhead. Sections were blocked in 0.5% BSA in PBS and incubated in a 1:250 dilution of antibody 5A2. Following washes, the monoclonal antibody was labeled with a 1:250 dilution of goat-anti-mouse IgG antibody coupled to 10nm colloidal gold beads which appear as black dots on the photomicrographs. The label was fixed with glutaraldehyde, and the sections were counterstained with uranyl acetate. All bars = 0.1  $\mu$ m.

Figure 8.

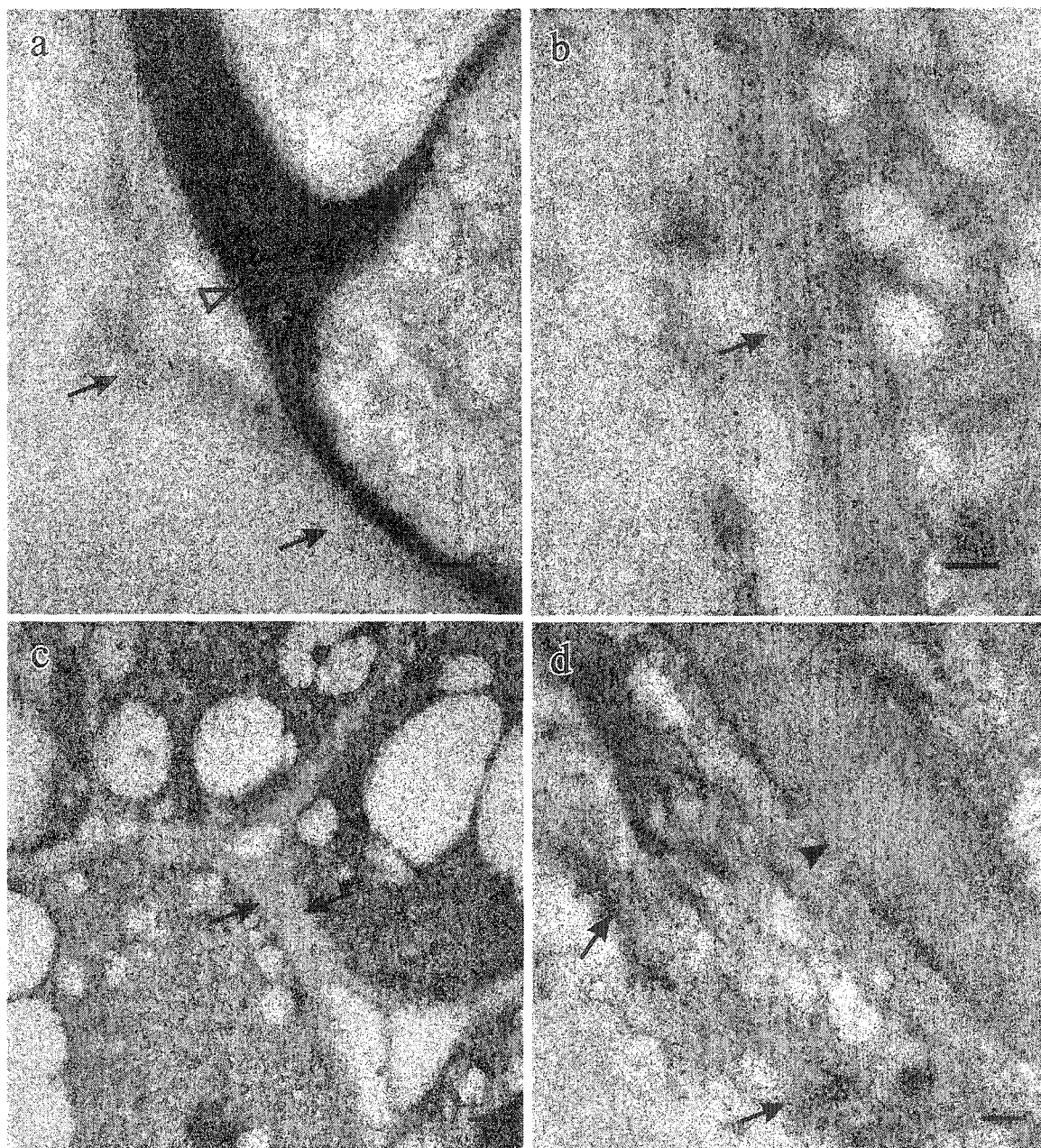


Figure 9.

Immunogold electron microscopy of *P. marinus* prezoosporangia formed by thioglycollate incubation (a-c), or zoosporangia by thioglycollate and artificial sea water incubation (d). Arrows indicate the 5A2-reactive cell wall which, if present, is loosely attached (b, d) or completely separated (c) from the non-labeled prezoosporangium or zoosporangium wall (arrowhead) which overlies the plasma membrane (open arrowhead). The cell walls of forming zoospores (d) are not labeled by antibody 5A2 (open arrow). Sections were blocked in 0.5% BSA in PBS and incubated in a 1:250 dilution of antibody 5A2. Following washes, the monoclonal antibody was labeled with a 1:250 dilution of goat-anti-mouse IgG antibody coupled to 10nm colloidal gold beads which appear as black dots on the photomicrographs. The label was fixed with glutaraldehyde, and the sections were counterstained with uranyl acetate. Bars are 0.5  $\mu\text{m}$  (a, b, d) or 1  $\mu\text{m}$  (c).



Figure 9.

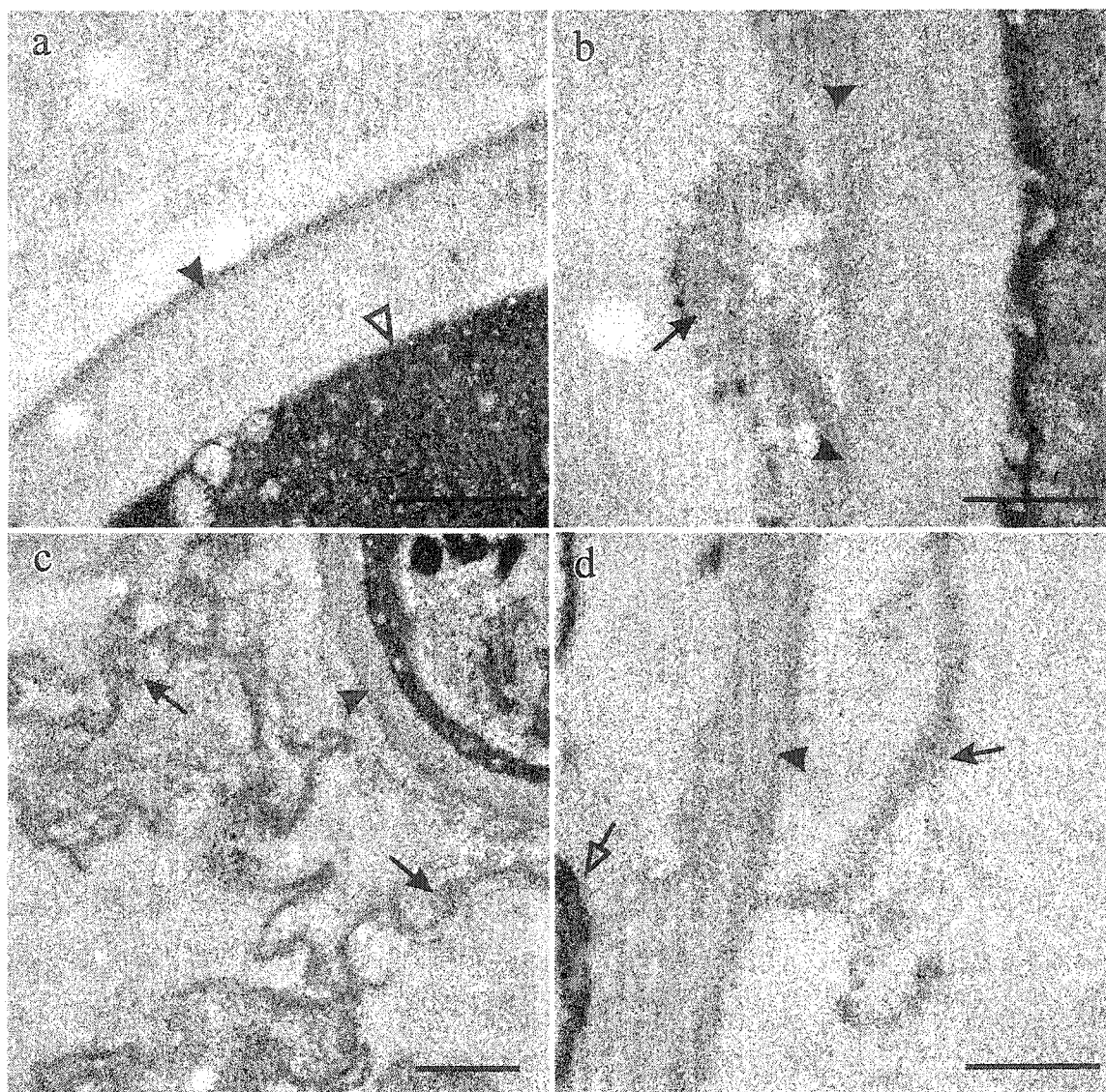
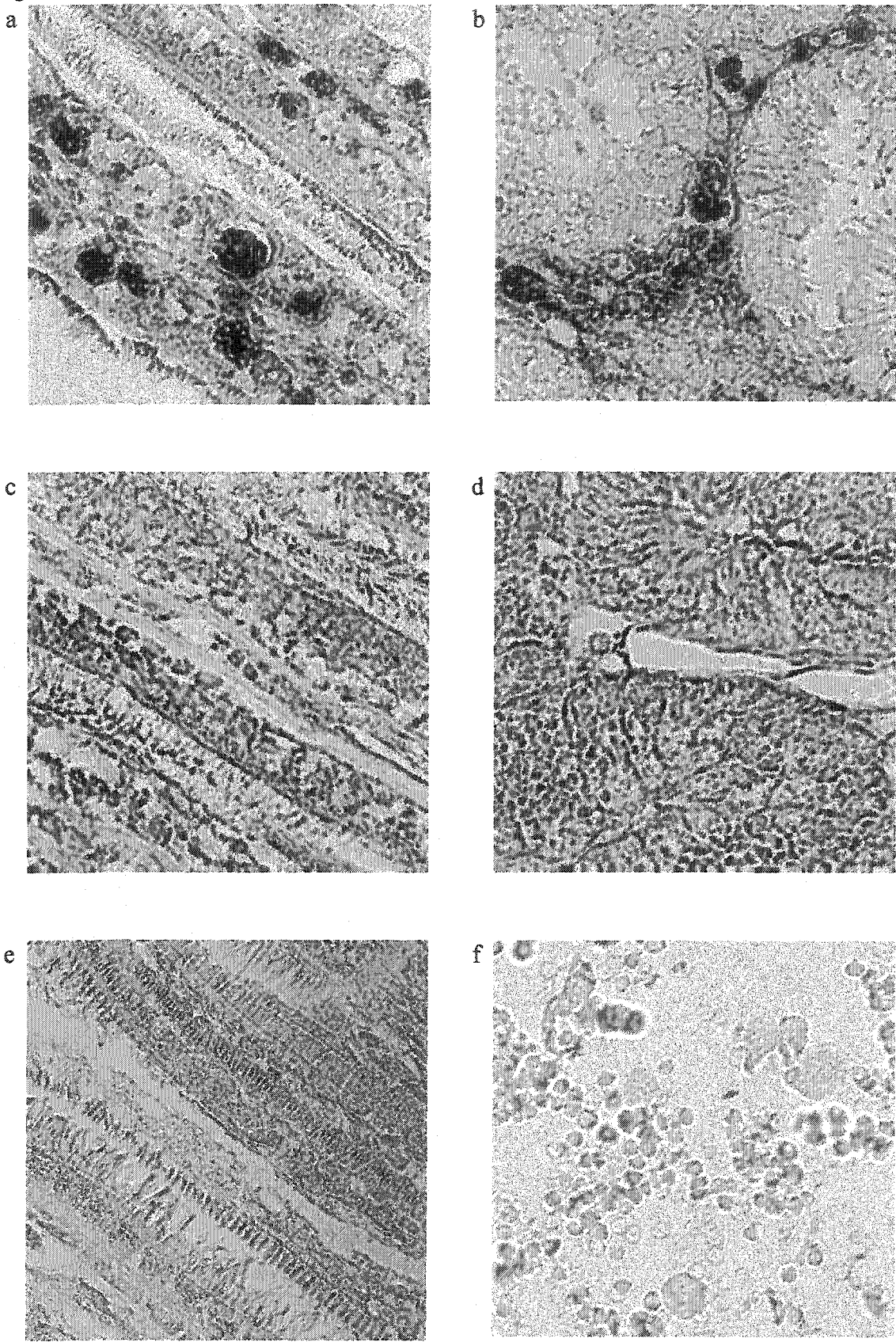




Figure 10.

Immunohistochemical detection of *P. marinus*. Tissue sections from the gill (a, c) or digestive gland (b, d) of *C. virginica* oysters infected (a, b) or not infected (c, d) with *P. marinus*, or from *P. atlanticus*-infected *Ruditapes decussatus* gill (e) or *Hematodinium perezii*-infected *Callinectes sapidus* hemolymph (f). Tissue sections were deparaffinized in xylene, rehydrated through an ethanol series, blocked with 3% BSA in TTBS, and incubated for 1 hour in a 1:500 dilution of purified antibody 5A2. Following three washes, the bound antibody was labeled with a 1:250 dilution of a peroxidase-coupled, Fc-specific goat-anti-mouse IgG. The section was again washed three times, and the peroxidase label was visualized by the chromogenic conversion of aminoethylcarbazole. The sections were counterstained with 1% Fast green for contrast.

Figure 10.



### Immunohistochemistry

Only antibody 5A2 labeled *P. marinus* in fixed, embedded oyster tissue sections. In these sections, both single trophozoites as well as multicellular tomites were clearly visible, and the staining of these cells was both intense and specific (Fig. 10a, b). No reactivity was seen in uninfected oyster tissues (Fig 10c, d). There was also no crossreactivity with *Perkinsus atlanticus* (Fig. 10e) or with the dinoflagellate *Hematodinium perezii* (Fig. 10f).

### Discussion

*Perkinsus marinus* secretes a complex set of extracellular products by apparently disparate mechanisms, including release from the cell wall and secretion from vesicles (Fig. 7). It is not known how the ECP present in vesicles are transported extracellularly, though there is evidence of both lomasome-type membranous bodies occurring near the cell wall, as well as micropores which open to the wall (Sunila et al., 2001). The pattern of recognition by monoclonal antibodies indicates that the numerous ECP may originate from a much smaller number of parental molecules (Fig. 1). The single epitope specificity of antibody 5A2, for example, is able to recognize at least nine different molecular weight forms, 9E12 and 1B3 three forms each, and 5G9, two forms. While antibody 5H11 recognized a single band, its failure to label any cellular structure may indicate that it is simply a degradation product of another parent molecule. In no case does the detection of multiple bands appear to be due to shared glycosylation moieties (Fig. 3).

The protein detected by antibodies 5G9 and 1B3 is detected *in situ* only in vesicular structures at early time points, possibly only while the culture is actively replicating. Even then, it is only detected in a subset of cells and, *in situ*, only by antibody 1B3 (Fig. 7a). There are several possible explanations for this behavior. Both antibodies recognize epitopes which are apparently dependent on protein conformation,

rather than the linear amino acid sequence; 1B3 does not recognize denatured protein, and neither antibody detects the protein following disulfide reduction. This likely explains the failure to detect this protein in fixed cell preparations and tissues. Structural alterations of the protein may take place within the secretory vesicles or following release from the cell which affect antibody detection. The intracellular recognition of antigen by 1B3 may result from detection of an immature protein form. Immunoaffinity purification of the ECP products recognized by 1B3 demonstrates that, in addition to the p107/p102 doublet recognized by both antibodies, there is recognition of an 81 kDa protein. While the structure of p107/p102 is not clear, under reducing conditions the affinity purification products of antibodies 1B3 and 5G9 appear as a band of approximately 42 kDa mass. This presents the possibility that p81 is a dimeric form of p42, and is the form detected by 1B3 *in situ*. It is uncertain how this protein then assembles into the 107 kDa form detectable by both antibodies, since there are no other protein bands visualized on reducing SDS-PAGE to account for the added mass. In this model, 5G9 recognition is dependent on the structure of the fully assembled molecule.

When the p42 subunit of p107/p102 is subjected to two dimensional electrophoresis (Fig. 5), there is evidence of minor mass variation, and post-translational modification. The approximate 1 kDa mass variation could be due to a difference in protein structure or glycosylation, and may account for the formation of the doublet under non-reducing conditions. The major isoelectric point variations may be due to differences in phosphorylation or to the presence of charged sugar residues on the molecule (Mann and Jensen, 2003). If these isoelectric differences are due to phosphorylation, this may indicate differences in protein activation among the various isoforms.

The function of the p107/p102 molecule is not known, and the peptide sequences from tandem mass spectroscopic analysis did not allow functional prediction. Due to its apparent production during log-phase *in vitro* growth (Fig. 7a), it may play a role in the

early infection process, or in stimulation of cell division. *Perkinsus marinus* cells grow more rapidly when exposed to conditioned medium; presumably due to the presence of a secreted growth factor (Gauthier and Vasta, 1995; Bushek et al., 2000). The amount of p107/p102 present in culture supernatants is depressed when the cultures are grown in the presence of whole oyster homogenates (Fig. 6a), or homogenates of individual oyster tissues or organs (See this dissertation, Chapter 3). This may be due either to destruction or modification of the protein during or after its secretion into the culture medium, or to downregulation of expression of that protein under those medium conditions. The preservation of detection of the protein in ECP-spiked homogenates indicates that the latter is more likely (Fig. 6b). Downregulation of protein expression in the presence of homogenates could be the consequence of an oyster defense mechanism, especially if the protein is a virulence factor. This seems less likely, however, given the increased infectivity of cells grown in supplemented medium (Earnhart et al., 2004). An alternative explanation is that p107/p102 is not downregulated in the presence of supplements, but upregulated in their absence. The p107/p102 protein may be secreted under the stressful condition of culture in an unsupplemented, protein-free medium. If true, it may be involved in nutrient transport or scavenging of scarce nutrients such as iron (Gauthier and Vasta, 1994).

In contrast with the time-dependent, vesicular location of the p107/p102 antigen recognized by antibody 1B3, the immunofluorescent staining pattern of antibody 9E12 demonstrated a markedly different pattern of intracellular processing or storage. The protein recognized by 9E12 was apparently constitutively produced, and the pattern of staining indicated that it was present within large intracellular compartments joined by a filamentous network, possibly representing the endoplasmic reticulum and/or Golgi complex (Soltys et al., 1996). This localization could not be confirmed ultrastructurally. The failure of antibody 9E12 in immunogold staining was likely due to the inability of the epitope to withstand fixation and embedding. It is not known whether the three

molecular weight forms (Fig. 1) are the result of extracellular cleavage or if they are related proteins. The decreasing amount of material with decreasing molecular weight seems, however, to indicate that the two lower molecular weight forms are the products of degradation of the parent molecule (Fig 1).

Antibody 5A2 recognizes a series of proteins, the largest of which has a molecular weight similar to the *Perkinsus* wall protein, PWP-1, described by Montes et al. (2002). Antibodies against PWP-1 labeled the walls of mature trophozoites, immature trophozoites encased within a parental tomont wall, and the thickened wall of prezoosporangia. In this study, antibody 5A2 labeled both trophozoite and tomont cell walls (Fig. 7d, e) but did not label the apparently structurally dissimilar cell wall of the prezoosporangia (Fig. 7f). As the trophozoites enlarged in thioglycollate medium, there was a change in the cell wall structure such that it stained with Lugol's iodine (Fig. 7g) (Ray, 1952). As this transition occurred, the amount of 5A2 label was greatly reduced, and its distribution was patchy (Fig. 7f), apparently due to shedding of the loosely attached 5A2-positive cell wall layer with the formation of a new cell wall. These findings were confirmed ultrastructurally. Shedding of the outermost cell wall material appeared to occur to some extent in trophozoites (Fig 8a, b), and cells transitioning to the prezoosporangium stage almost completely released this outer layer from the underlying wall, leaving only occasional adherent patches (Fig. 9). A similar immunogold staining pattern was seen in trophozoites grown in the presence of oyster homogenate, with the walls of some cells having a two-layered morphology consisting of the fibrillar, 5A2-positive outer wall covering a more electron dense, lightly labeled inner wall (Fig 8d). Oyster tissue supplementation has been noted to alter cell morphology to more closely resemble the prezoosporangium stage induced by thioglycollate incubation, including cellular enlargement and formation of a thickened cell wall (Earnhart et al., 2004). The formation of an antigenically dissimilar inner wall during supplementation supports the contention that oyster product supplements causes a partial transition to the

prezoosporangium life stage, and may offer insights into the *in vivo* signals or conditions required for this transition. The antigenic differences between these walls may also explain the failure of some antibodies created using prezoosporangium antigen to detect other *P. marinus* life stages (Choi et al., 1991; Dungan and Roberson, 1993).

Despite the binding similarities between the anti-PWP-1 antibody and 5A2, there are marked epitope differences, assuming they label the same parental protein. While the anti-PWP-1 antiserum recognized both *P. atlanticus* and *P. marinus* protein (Montes et al., 2002), antibody 5A2 was specific for the wall of *P. marinus*. Furthermore, despite polyspecific recognition by the anti-PWP-1 antiserum, there was only a single 233kDa band recognized by immunoblot, in contrast with the series of bands recognized by 5A2. The detection of a protein series by antibody 5A2 may indicate either an incidental or functional degradation of a parent molecule. PWP-1 protein has been demonstrated to be resistant to proteolytic degradation by the serine protease trypsin (Montes et al., 2002), suggesting that the apparent processing of the protein detected by 5A2 is due to a protease with a specific ability to degrade this structural wall protein. *Perkinsus marinus* secretes numerous serine proteases into the culture medium (LaPeyre and Faisal, 1995; LaPeyre et al., 1995; Garreis et al., 1996; Faisal, Schafhauser et al., 1999), some of which can be modulated by the environment in which the cells are grown (See this dissertation, Chapter 3; MacIntyre et al., 2003; Earnhart et al., 2004). It is possible that one or more of these proteases functions in the processing of cell wall components. Faisal et al. (1999) demonstrated that the cyclopeptide protease inhibitor bacitracin was able to inhibit *P. marinus in vitro* growth, indicating that the appropriate functioning of the serine proteases is critical for parasite survival.

The proteolytic processing of wall components to forms having immune-modulatory or other virulence activities occurs in bacterial pathogens, as well as in other protozoan parasites. The bacterial fish pathogen *Renibacterium salmoninarum* regulates its major extracellular product, p57, by cleavage of a cell-bound form, and subsequent

degradation into a series of fragments by endogenously produced serine proteases (Rockey, Gilkey et al., 1991; Rocky, Turaga et al., 1991; Wiens et al., 1999). The malarial parasite *Plasmodium falciparum* proteolytically alters cell surface proteins during maturation (Rosenthal et al., 1987; Braun-Breton and Pereira da Silva, 1988) and addition of protease inhibitors, while not altering the cleavage of the parental molecule, diminishes the rate of loss of the antigens into the culture medium (Lyon and Haynes, 1986). Diffuse staining in the tissues surrounding the cells indicates that the 5A2 antigen is not confined to the *P. marinus* walls during active infection, but may be shed into tissues (Fig. 10a, b). This pattern is similar to the diffuse immunofluorescent staining noted by Dungan and Roberson (1993) in infected tissue sections stained with a polyclonal antiserum.

The biochemical composition of the *P. marinus* cell wall is not known, but may have similarities with the glycoprotein exoantigens of the apicomplexan *Cryptosporidium parvum* (Riggs et al., 1997), the secreted glycoprotein of *Toxoplasma gondii* (Zhang et al., 2001), and to the glycoprotein cell wall found in some yeasts (Nelson et al., 1991). Yeast cell walls are composed of several types of carbohydrate and mannan glycoproteins (Nelson et al., 1991). In *Leishmania* parasites, mannan is a virulence factor critical for parasite survival within macrophages (Ralton et al., 2003). The major surface glycoproteins of *Trypanosoma cruzi* are ligands of the mannose-binding proteins in the human host and may function as mediators of adhesion with host cells (Kahn et al., 1995; Kahn et al., 1996).

*Perkinsus marinus* ECP has been found in previous studies to be highly suppressive of the murine humoral immune response (Earnhart and Kaattari, 2003). If the *P. marinus* cell wall has functional similarities to the mannan cell wall in the yeasts, this could offer a possible explanation for the immunomodulatory effects of *P. marinus* ECP in both the mammalian and the oyster systems. Mannose, along with several other sugars, has been documented by lectin labeling to occur on the *P. marinus* cell surface



(Brown and Faisal, 1998; Gauthier et al., 2002). Mannans have several immunomodulatory activities, some of which may impact on oyster defense functions. These include induction of prostaglandin E<sub>2</sub> secretion by monocytes, induction of suppressor T-lymphocytes, suppression or induction of proliferation of B-lymphocytes, suppression of T-lymphocyte proliferation, alteration of normal cytokine responses, interference with leukocyte homing, activation of complement, and inhibition of myeloperoxidase activity (Mikami et al., 1982; Wright et al., 1983; Podzorski et al., 1989; Nelson et al., 1991; Krizkova et al., 2001). Garreis et al. (1996) described several dysfunctions in the oyster defense system which appeared to be mediated by the ECP of *P. marinus*, including a reduction in both random and chemotactic migration of hemocytes, and a decrease in the titer of agglutinins and lysozyme in the plasma. There is also an abrogation by *P. marinus* cells and ECP of the oyster hemocyte myeloperoxidase-mediated (Austin and Paynter, 1995) reactive oxygen species response (Volety and Chu, 1995; Anderson, 1999). If the release of these cell wall components is mediated by the degradation of the 5A2 parental antigen, the appearance of degradation products may signify activation of a virulence mechanism.

An understanding of the mechanisms of ECP regulation, storage, and secretion offers new insights into potential virulence mechanisms of this protozoan parasite. Both the constitutive expression and release, and the storage and time-dependent release of products indicates the diversity of functions that the ECP likely mediate. The *in vitro* study of these dynamics in host product-supplemented medium has demonstrated altered expression of the p107/p102 protein as well as the formation of a structurally and antigenically distinct cell wall. Previous studies have shown increased parasite infectivity under these conditions, making these changes particularly interesting. The ability of *P. marinus* to survive and replicate within oyster hemocyte phagosomes indicates that there are one or more mechanisms by which *P. marinus* subverts the host defense. The ECP storage in and release from the cell wall, potentially by the action of

specific serine proteases, is conceptually an efficient means by which to mediate that survival. The sloughing and loose adherence to the plasma membrane of the trophozoite cell wall may indicate that it is less a structural component than a mediator of the host-parasite relationship. The loss of 5A2 antigen detection in the more stable, and likely structurally important prezoosporangium wall further supports this possibility.

Increasingly, the ECP appear to be an important target not only for investigations of virulence mechanisms, but in the study of basic processes in this protozoan parasite.

## Chapter 7

### Summary

The modulation of *Perkinsus marinus* morphology and function through *in vitro* culture with host-derived supplements has demonstrated new aspects of the complexity of the host-parasite relationship. Despite the complexity of these interactions, this research has shown that parasite functions likely mediated by host signals can be selectively modulated by host-derived extracts and studied *in vitro*. The most promising of these supplement-induced alterations is the reversal of *in vitro* culture-induced attenuation. The concurrent study of attenuated and virulent forms of a clonal isolate is an ideal tool for the investigation of potential virulence factors and activities. This, combined with the development of specific antibodies to precisely track and quantify some of the supplement-induced changes, has provided new information regarding the regulation of extracellular proteins, proteases, the sources of potential extracellular product (ECP) virulence factors, the inductive environment required for transition from binary to palintomic parasite reproduction, the regulation of parasite proliferative rate, and the host-induction of, and structural changes associated with, the transition from trophozoite to zoosporangium.

The reversal of parasite attenuation was accomplished by medium supplementation with oyster plasma or whole oyster homogenates. Increased infectivity was specifically induced by oyster tissues, though it was not related to the disease susceptibility of the oyster from which the supplement was derived. This implies that any differences in host susceptibility to the parasite are independent of the mechanisms involved in regulation of parasite infectivity. Supplementation also elicited other changes

in the parasite, including the protease secretion pattern, parasite size, and morphology. None of these changes, however, absolutely correlated with the increased infectivity. The complex set of phenotypic changes caused by supplementation appeared, in some cases, to be differentially modulated by homogenates derived from different oyster populations or species. There were also variations in infectivity, morphology, and protease production in different *P. marinus* cell lines. While these experiments did not seek to address co-adaptation between local host and parasite populations, these combined differences could indicate this possibility.

The proliferative, morphological, and ECP changes caused by medium supplementation were elicited to varying degrees by each supplement derived from specific organs or tissues. This tissue-specific modulation of parasite functions may play an important role in the regulation of parasite growth, life stage transition, and other functions. Parasite proliferation was reduced by medium supplementation, with two exceptions. Plasma-supplemented cultures had somewhat reduced proliferation, but not to the same extent seen with other supplements, while supplementation with adductor muscle was associated with proliferation exceeding that of the control cultures. The parasite multiplies rapidly within oyster hemocytes and in the interstitial spaces of the open circulatory system, and exposure to plasma or the highly vascularized adductor muscle may induce a proliferative response in the parasite. Alternatively, there may be, in plasma and adductor muscle, a lack of down-regulatory signals for proliferation which occur in the other oyster tissues. This hypothesis is supported by the reduction in proliferation from control levels that occurs in the presence of other tissue supplements. Downregulation of proliferation in the presence of large amounts of certain degraded tissues may represent a parasite adaptation to prolong host survival.

The proliferative response is not, however, the only measure of parasite growth in culture. Supplementation, especially with whole oyster homogenate, also caused marked cellular enlargement. This enlargement was specifically induced by oyster products and

was similar to the induction of increased infectivity, in that it was independent of host susceptibility. At lower supplementation levels, the increase in cell size was not sufficient to counteract the effect of decreased proliferative rate on the aggregate cell volume; however, at some moderate to high supplementation levels, there is a net increase in this aggregate volume, indicating some form of parasite growth or transformation, even at drastically reduced proliferative rates. While this effect was noted to some extent in cultures supplemented with homogenate of *Crassostrea ariakensis* or *C. gigas*, the aggregate cell volume was never as great as with *C. virginica* supplements, probably due to progressive loss of culture viability at higher supplement doses. The ability to maintain lower parasite burden and aggregate parasite volume in *C. ariakensis* and *C. gigas* may partially explain the increased tolerance of *P. marinus* infection in these oyster species.

A subset of the cells in *C. virginica*-supplemented cultures appeared by light microscopy to have a thickened cell wall. This, combined with the drastic size change, was thought to resemble the transition to prezoosporangia typically induced by thioglycollate incubation of trophozoites. The morphologic similarity between the supplemented and thioglycollate treated cells was further supported by the binding pattern of a trophozoite wall-specific monoclonal antibody during immunogold electron microscopy. The transition to an antigenically and structurally distinct cell wall form was found in both situations, indicating medium supplementation can be used to more carefully investigate the signals or conditions that induce this life stage transition. This stage is typically seen *in vivo* only in moribund oysters; therefore, rather than a specific inductive molecule, it is logical to assume that the parasite may simply be affected by a large amount of degrading oyster tissue, possibly by the concentrations of free lipids or amino acids, or by the alteration in the reduction-oxidation state in the local environment. Thioglycollate itself is a reducing agent and creates an anaerobic condition during incubation. The size and structural changes elicited by whole oyster homogenate could

not be replicated by supplementation with any specific oyster tissue, even when the supplement was derived from the digestive gland and gonad, which is essentially the bulk of the oyster. Combinatorial studies may be helpful in elucidating the exact requirements for induction of this transition.

Supplementation also caused a remarkable change in the mechanism of *in vitro* proliferation. Unsupplemented cultures replicate, apparently exclusively, by binary fission. Culture medium supplementation, especially with homogenates of whole oyster and digestive gland/gonad, resulted in the bulk of replication occurring by palintomy within parental tomons. Tomonts are very commonly seen in infected tissue sections, and thus appear to be the normal replicative mechanism. The transition to binary fission in the absence of host products is puzzling, but may be due to the small size of the cells in unsupplemented cultures. While palintomy, by definition, does not require a proportional increase in cytoplasmic volume prior to karyokinesis and cytokinesis, there may be an absolute limit to the cytoplasmic volume that enables multiple fission. The cellular enlargement that occurs with supplementation may thus enable the parasite to revert to its normal reproductive mechanism.

The extracellular products of *P. marinus* have been shown to be immunomodulatory in both the oyster and, in this research, the mouse. While the mechanism of immune dysfunction is not clear, it may be due to modulation of shared innate immune mechanisms. The availability of monoclonal antibodies for affinity purification will allow investigation of the effects of isolated ECP components on innate immune function. Antibodies produced against ECP have shown that they are heterogeneous in their manner of storage and secretion. This implies heterogeneity of function. There are clearly modulations in ECP during culture supplementation, and some of these may play a role in the supplement-induced alteration of virulence. The failure of subtractive immunization techniques to distinguish novel ECP antigens from cells exposed to oyster supplements may indicate that any role of ECP in increasing

infectivity and virulence may be due simply to alterations in the expression level of an ECP component. This type of alteration is clearly seen in quantification of the p107/p102 protein; with any medium supplementation, there is apparent downregulation of protein production. This also occurs with some serine proteases during medium supplementation. In many cases when there is increased low molecular weight protease activity, there is also decreased expression of some high molecular weight proteases. While each of these proteins may be independently regulated, it is also possible that the upregulated low molecular weight proteases are simply modifications of extant proteins caused by the medium supplementation conditions. The available panel of monoclonal antibodies has already demonstrated that the pattern of ECP is deceptively complex. Many of the molecules present may be degraded components of relatively fewer parental molecules.

If the low molecular weight proteases are formed by processing of higher molecular weight forms, there are apparently very specific culture conditions required to initiate and stabilize those changes. Supplementation with whole *C. virginica* homogenate, while the most reliable method for low molecular weight protease induction, is not always effective, and there is an effect of dose with all supplement types. When supplementing with individual organs, both adductor and heart muscle homogenates effectively induced low molecular weight proteases, but the optimal inductive concentration varied widely between these two muscular tissues. The induction by those tissues also did not appear to be associated with the high molecular weight downregulation often seen with whole oyster supplemented cultures. This implies that the low molecular weight proteases are induced molecules, rather than products of processing within the ECP, and may have a specific function associated with the *in vivo* correlate of those medium conditions. In addition, the results of non-zymographic assessment of protease activity indicate that there may be other proteases induced by

exposure to specific tissues, especially the gill and mantle. This is particularly interesting, as these tissues are a primary entry point for the parasite.

This research has provided some of the initial information necessary for determination of mechanisms of infectivity and parasite alteration of host defense responses. It has investigated potential *in vitro* correlates of host-induced modulation of parasite infectivity, reproduction, morphology, life stage transition, and protein and protease secretion. Progress has also been made toward resolution of the exact mechanisms of induction involved in those changes, with candidate molecules in some cases resolved to the tissue level. In addition, it has provided a number of antibody tools for the continued study of the host-parasite interaction, both *in vitro* and *in vivo* which will facilitate the tracking and quantification of molecules of potential importance to the diseases process.



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## VITA

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